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UNIVERSITY OF ALBERTA

WHEY PROTEINS: SEPARATION AND THERMAL BEHAVIOUR RELEVANT TO WHEY PROCESSING

BY

GEORGE PATOCKA

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

IN

FOOD PROCESSING

DEPARTMENT OF FOOD SCIENCE

EDMONTON, ALBERTA

SPRING 1990

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ABSTRACT

The objective of this project was to study the effects leading to better whey protein separation from whey by ultrafiltration (UF) and to characterize further thermal behaviour of individual whey proteins relevant for industrial applications.

Centrifugal clarification of cottage cheese whey was found to be an effective pretreatment enhancing ultrafiltration performance.

Sweet cheese wheys acidified below pH 4 showed slightly lower resistance towards heat induced protein aggregation than similarly treated acid wheys due to lower total calcium content.

The heat resistance of isolated whey proteins, β-lactoglobulin (β-lg), α-lactalbumin (α-la) and serum albumin (SA) was studied in pH-adjusted UF permeates of cottage cheese whey with regular or reduced Ca content. The β-lg was heat-stable at pH 3.8-3.6 depending on the Ca content and coagulated rapidly at higher pH values except 6.5-7.0 in permeates with low Ca content. The α-la showed no visible coagulation upon heating within the pH range of 3.5-5.0 in regular and 3.5-7.0 in decalcified permeate, respectively. The SA coagulated at pH 4.2 and above. In mixtures of whey proteins, some co-precipitation of α-la and/or SA with the predominant β-lg was observed regardless of Ca content.

A separate study showed that both α-la and β-lg are capable of binding calcium. No Ca-α-la and Ca-β-lg interactions were observed below pH 4 and 5,

respectively. The increase of ionic strength had a negative effect on Ca-protein binding with both whey protein fractions. The trend of binding characteristics of 0.2% α-la and 0.4% β-lg observed in UF permeate was similar to that observed in simple buffer solutions.

As one possible industrial application, rennet and acid whey ultrafiltration retentates (UFR) were combined with skimmilk in various ratios. Upon heating at 93°C milk blends containing acid whey UFR showed low heat stability, when casein : whey protein ratio was less than 70/30. No heat induced coagulation was observed for blends of skimmilk with low Ca wheys, including rennet whey UFR or decalcified acid whey UFR.

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In the final preparation of this manuscript, I acknowledge with sincere gratitude the advice and critical review of my examining committee, especially Drs. C.V. Morr, P. Sporns and F. Temelli.

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Table of Contents

Chapter Page

1. INTRODUCTION

1.1. Current status of industrial utilization of whey and whey proteins.......... 1
1.2. Research objectives.......... 3
1.3. Experimental investigations: methodology, results, discussion.......... 4
1.4. References.......... 5

2. LITERATURE REVIEW.......... 7

2.1. Whey protein distribution.......... 7
2.1.1. β-lactoglobulin.......... 9
2.1.2. α-lactalbumin.......... 11
2.1.3. Other whey proteins.......... 13

2.2. Ionic equilibria in whey.......... 14
2.2.1. Complexation effects.......... 18

2.3. Membrane separation of whey proteins.......... 24
2.3.1. Membrane fouling.......... 26
2.3.2. Whey pretreatment for membrane processing.......... 28

2.4. Thermal behaviour of whey proteins.......... 30
2.4.1. Isolated β-lactoglobulin.......... 31
2.4.2. Isolated α-lactalbumin.......... 34
2.4.3. The effects of environmental conditions on whey protein thermostability.......... 35
2.4.4. Heat induced whey protein interactions.......... 38

2.5. References.......... 41

3. RAPID CLARIFICATION OF COTTAGE CHEESE WHEY BY CENTRIFUGATION AND ITS CONTROL BY MEASUREMENTS OF ABSORBANCE.......... 50

3.1. Introduction.......... 50

3.2. Materials and Methods.......... 51

3.3. Results and Discussion........ 52

3.4. Conclusions........ 54

3.5. Tables and Figures........ 55

3.6. References........ 59

4. PROTEIN COAGULATION IN SWEET AND ACID WHEYS UPON HEATING IN HIGHLY ACIDIC CONDITIONS........ 60

4.1. Introduction........ 60

4.2. Materials and Methods........ 62

4.3. Results and Discussion........ 64

4.4. Conclusions........ 67

4.5. Tables and Figures........ 69

4.6. References........ 75

5. HEAT STABILITY OF ISOLATED WHEY PROTEIN FRACTIONS IN HIGHLY ACIDIC CONDITIONS........ 76

5.1. Introduction........ 76

5.2. Materials and Methods........ 77

5.3. Results and Discussion........ 79

5.4. Conclusions........ 82

5.5. Tables and Figures........ 84

5.6. References........ 92

6. EFFECT OF pH AND CALCIUM ON THERMAL BEHAVIOUR OF ISOLATED WHEY PROTEINS.............. 93

6.1. Introduction.............. 93

6.2. Materials and Methods.............. 95

6.3. Results and Discussion.............. 96

6.4. Conclusions.............. 100

6.5. Tables and Figures.............. 101

6.6. References.............. 107

7. BINDING OF CALCIUM TO ISOLATED WHEY PROTEINS.............. 109

7.1. Introduction.............. 109

7.2. Materials and Methods.............. 110

7.3. Results and Discussion.............. 116

7.4. Conclusions.............. 119

7.5. Tables and Figures.............. 121

7.6. References.............. 127

8. HEAT INDUCED COAGULATION IN MILK WITH MODIFIED CASEIN : WHEY PROTEIN CONTENT.............. 130

8.1. Introduction.............. 130

8.2. Materials and Methods.............. 131

8.3. Results and Discussion.............. 133

8.4. Conclusions.............. 137

8.5. Tables and Figures.............. 138

8.6. References.............. 148

9. SUMMARY AND CONCLUSIONS.. 150

9.1. Summary of research findings... 150

9.2. Significance for industrial processing.. 154

9.3. Recommendation for future studies... 156

List of Tables

Table Page

1-1. Average composition data for sweet and acid types of wheys. 2

2-1. Variations in amino acid composition of genetic variants of β-lg. 10

2-2. Dissociation constants of important acids present in whey. 16

2-3. Formation constants for Ca and Mg citrate complexes. 22

2-4. Thermodynamic parameters of whey protein heat denaturation. 32

3-1. The amount of casein fines and the corresponding absorbance index values for cottage cheese whey. 55

4-1. The composition and heat stability (critical pH range) of different types of industrial and laboratory prepared wheys investigated. 69

5-1. Total solids, total nitrogen, calcium and phosphorus content of regular and ion-exchange treated cottage cheese whey UF permeate. 84

6-1. Total Ca content in pH adjusted UF permeates and initial turbidity of solubilized proteins. 101

7-1. Binding maxima of Ca to β-lg at various Ca concentrations. 121

8-1. Heat stability of mixtures of skimmilk and cottage cheese whey UF. 139

8-2. Calcium levels and observed heat stability (time before coagulation at 93°C) of UFR in comparison to skimmilk. 140

8-3. Calcium content of whey UF permeates and thermal stability (time before coagulation at 93°C) of skimmilk/whey UFP blends. 141

List of Figures

Figure Page

2-1. Whey protein distribution in cheese whey. 8

2-2. A schematic representation of cheese whey ultrafiltration. 25

3-1. Sedimentation profile for casein fines in cottage cheese whey. 56

3-2. Relationship between the casein fines content and absorbance at 900 nm for cottage cheese whey. 57

3-3. Ultrafiltration flux of cottage cheese whey with various clarity levels filtered on DDS Lab-20 module. 58

4-1. Changes in turbidity (absorbance index) of acid (a) and sweet (b) wheys upon heating at 92°C at various pH conditions. 70

4-2. Turbidity (absorbance index) changes in acid wheys heated at 92°C with various amounts of EDTA (a) or citric acid (b). 71

4-3. Heat-induced turbidity changes in sweet whey with 2:1 excess EDTA or citric acid based on Ca^{2+} equivalents. 72

4-4. Effect of Ca^{2+} replacement by Na on heat-induced turbidity changes in acid (a) and sweet (b) wheys. 73

4-5. Changes in turbidity of industrial wheys heated for 10 min at 92°C and varying pH conditions. 74

5-1. Heat stability of 0.4% β-lg solution in cottage cheese whey UF permeate with the normal and reduced Ca content. 85

5-2. SDS-PAGE electrophoretograms of 0.4% β-lg solution in cottage cheese whey UF permeate with normal (A) and reduced (B) Ca content. 86

5-3. Heat stability of 0.2% α-la and 0.1% SA solutions in cottage cheese whey UF permeate with normal and reduced Ca content. 87

5-4. SDS-PAGE electrophoretograms of 0.2% α-la solution in cottage cheese whey UF permeate with normal (A) and reduced (B) Ca content. 88

5-5. SDS-PAGE electrophoretograms of 0.1% SA solution in cottage cheese whey UF permeate with normal (A) and reduced (B) Ca content. 89

5-6. SDS-PAGE electrophoretograms of 0.1% SA, 0.2% α-la and 0.4% β-lg mixture in cottage cheese whey UF permeate. 90

5-7. SDS-PAGE electrophoretograms of cottage cheese whey before (A) and after (B) Ca ion exchange treatment 91

6-1. Heat stability of 0.2% α-lactalbumin in cottage cheese whey UF permeate with normal (A) and reduced (B) Ca content. 102

6-2. The effect of pH on the heat stability of 0.2% α-la in cottage cheese whey UF permeate heated for 30 min at 93±1^{o}C. 103

6-3. The heat stability of 0.4% β-lactoglobulin in cottage cheese whey UF permeate with regular (A) and reduced (B) Ca content. 104

6-4. SDS-PAGE electrophoretograms :
(a) 0.2% α-la and 0.4% β-lg solution in regular cottage cheese whey UF permeate heated for 30 min at 93±1^{o}C and (b) separated solubilized precipitates. 105

6-5. SDS-PAGE electrophoretograms :
(a) 0.2% α-la and 0.4% β-lg solution in decalcified cottage cheese whey UF permeate heated for 30 min at 93±1^{o}C and (b) separated solubilized precipitates. 106

7-1. The Ca-α-la binding profile at various pH in buffer solution at ionic strength = 0.08. 122

7-2. The Ca- β-lg binding profile at various pH in buffer solution at ionic strength = 0.08 123

7-3. The effect of pH and various ionic strength on binding of Ca to α-la. 124

7-4. The effect of pH and various ionic strength on binding of Ca to β-lg. 125

7-5. Binding of Ca to α-la (0.2%) and β-lg (0.4%) in UF permeate at various calcium concentrations. 126

8-1. SDS-PAGE patterns of heat-unstable and heat-stable milk with varying whey protein content before (a) and after heating (b) at 93°C for 30 min. 142

8-2. SDS-PAGE electrophoretograms of precipitates separated from milk with varying casein: whey protein content after heating at 93°C for 30 min. 143

8-3. The maximum whey protein content of milk blends stable upon heating at 92°C for 30 min. 144

8-4. Transmission electron micrographs of the cottage cheese whey UFR/skimmilk preparations after heating at 93°C for 30 min. 145

8-5. Transmission electron micrographs of the decalcified cottage cheese whey UFR/skimmilk preparations after heating at 93°C for 30 min. 146

8-6. Transmission electron micrographs of the unpasteurized rennet whey UFR/skimmilk preparations after heating at 93°C for 30 min. 147

1. INTRODUCTION

1.1. Current status of industrial utilization of whey and whey proteins

Whey is a dilute fluid resulting from the conversion of milk into cheese. Whey contains about 6.5% solids of which the major constituents are lactose (70-80%) and proteins (9-11%). The character of whey is determined by the way of casein separation from milk. The coagulation and separation of casein achieved by the addition of mineral acid or by acidification resulting from the acid generated by lactic acid bacteria produces the acid whey. Destabilization of casein micelles by milk-clotting enzymes with following casein precipitation yields the sweet whey. The sweet and acid wheys differ significantly in the level of residual acidity and overall mineral composition as shown in Table 1-1.

In recent years the environmental concerns, scarcity of milk and growing recognition of whey revenue potential has changed the status of whey from a dairy waste-product to a resource of valuable constituents utilized by the industry. The development of new technologies for the economically feasible isolation of proteins from the whey contributed to the increased use of whey proteins in processed foods (Marshall, 1982; Morr, 1982). Currently, membrane molecular separation in general, and ultrafiltration (UF) in particular are being widely adopted as industrial techniques for whey processing (Jelen, 1979).

Whey proteins are known to be highly susceptible to heat induced

Table 1-1. Average composition data for sweet and acid types of wheys[a].

<table>
<tr><th></th><th>sweet wheys
(pH 5.9-6.4)</th><th>acid wheys
(pH 4.6-4.7)</th></tr>
<tr><td></td><td colspan="2">composition
--------- (g/l) ---------</td></tr>
<tr><td>total solids</td><td colspan="2">63 - 70</td></tr>
<tr><td>protein</td><td>6 - 8</td><td>6 - 7</td></tr>
<tr><td>lactose</td><td>46 - 52</td><td>44 - 46</td></tr>
<tr><td>fat</td><td>0.2 - 1.0</td><td>0.2 - 0.5</td></tr>
<tr><td>calcium</td><td>0.4 - 0.5</td><td>1.3 - 1.6</td></tr>
<tr><td>magnesium</td><td>0.08</td><td>0.11</td></tr>
<tr><td>sodium</td><td colspan="2">0.4 - 0.5</td></tr>
<tr><td>potasium</td><td colspan="2">1.4 - 1.6</td></tr>
<tr><td>citrate</td><td colspan="2">1.4 - 1.7</td></tr>
<tr><td>phosphate</td><td>1 - 3</td><td>2 - 4.5</td></tr>
<tr><td>lactate</td><td>2.0[b]</td><td>6.4[b]</td></tr>
<tr><td>chloride</td><td>1.0</td><td>0.9</td></tr>
</table>

[a] adapted from Glass and Hedrick, 1976a, b; de Rham and Chanton, 1984; Hill, 1986; Jelen, 1979; Marshall, 1982; Walstra and Jenness, 1984

[b] no lactate in rennet or mineral acid whey

changes. As heating is a major technological treatment in dairy processing, the heat response of various whey systems has been the subject of substantial amount of investigation. Heat induced changes in whey proteins resulting in protein denaturation, aggregation and precipitation alter protein functional properties and bring about a reduction in solubility, emulsifying, foaming and thermosetting properties (Fox, 1982; Morr, 1982). The heat stability of whey proteins has a major implication for whey utilization (Mulvihill and Donovan, 1987).

The whey and whey proteins in particular are being used in a number of applications including fabricated foods such as dry soups, salad dressings, protein fortified pasta products, special dietary items, and ice cream. Use of whey protein products in infant formulas seems to be of worldwide significance. Utilization of liquid whey for manufacture of various alcoholic and nonalcoholic beverages has been succesfully commercialized by several processors (Jelen et al., 1987); however, whey beverages are still rare in most countries today. Recently, incorporation of whey proteins into the milk and marketing of subsequent dairy products gained attention in several European countries (Buchheim et al., 1986).

1.2. Research objectives

The principal research interest guiding this project was to investigate conditions for improved efficiency of whey protein separation during cheese whey ultrafiltration and to characterize further the thermal behaviour of individual whey proteins with the aim of identifying feasible industrial applications.

Specific objectives included:

(1) assesment of the UF flux improving effects of certain physical whey treatments;

(2) study of heat stability of whey proteins in various pH conditions and with varying calcium concentration;

(3) characterization of calcium interactions with the major whey proteins in whey systems of diverse chemical environment;

(4) investigation of the heat stability of milk with modified casein-whey protein ratio in relationship to the calcium content.

1.3. Experimental investigations: methodology, results and discussion

The body of this study consists of six manuscripts of technical papers that have been submitted for publication (2) or already published (3) in reputable journals; one paper has been published in a book.

The experimental work was divided as follows: (1) study of clarification efficiency aimed at casein fines removal from whey and its effectiveness for UF flux improvement; (2) study of heat precipitation of proteins in wheys treated according to 1 in highly acidic conditions; (3) evaluation of heat effects on isolated whey protein fractions under conditions identical to 2; (4) evaluation of thermal stability of whey protein isolates in systems described in 3, but in the pH region of 4.5-7.0; (5) assesment of interactions between calcium and whey proteins in conditions related to the previous studies (1-4); and (6) characterization of thermal behaviour of whey UF retentates alone and/or incorporated into the skimmilk, as one possible industrial application of these investigations.

Each of the six research activity areas is covered by a separate paper which contains description of methodology and analytical procedures, presentation and discussion of pertinent results in tables and/or graphs, as well as specific conclusions and applicable bibliography. The style and form of each paper follow individual requirements for publication. The footnotes at the first pages of the individual chapters indicate research personnel involved in this investigation, and the journal chosen for publication.

Some of the analytical work in this project (Ch. 4, 5, 7) was confirmed by analyses carried out by technical staff of the Alberta Agriculture Food Laboratory, Edmonton. The electron microscopy work (Ch. 8) was performed at the Electron Microscopy Centre, Agriculture Canada, Ottawa, by Dr. M. Kalab.

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2. LITERATURE REVIEW

2.1. Whey protein distribution

The whey contains from 5-7 g/l soluble proteins (Swaisgood, 1982). The proteins called whey or milk serum proteins are a heterogenous group; the approximate distribution of individual fractions is outlined in Fig. 2-1. The most recent review on the nomenclature of individual whey proteins and their genetic variants was given by Eigel et al. (1984). According to this classification the whey proteins consist of four principal fractions including β-lactoglobulin (β-lg), α-lactalbumin (α-la), serum albumin (SA) and immunoglobulins (Ig).

There have been numerous studies on characterization of individual whey proteins and much is known about their physico-chemical properties (McKenzie, 1971; Swaisgood, 1982). The dominantly globular conformation of all whey proteins is a factor contributing to their unique functional properties. Their susceptibility to denaturation, particularly by heat is a major consequence of the fragile stability of their three-dimensional structures.

The major fractions, β-lg and α-la, comprise about 75% of the whey proteins and tend to govern the properties of the total whey protein system. Our study was concentrated mainly on the behaviour of those two proteins under variable conditions common to whey systems during processing.

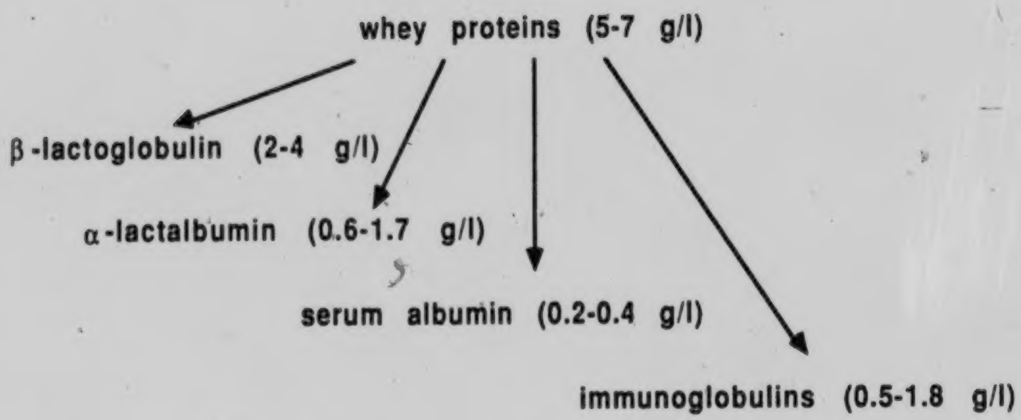
whey proteins (5-7 g/l)
β-lactoglobulin (2-4 g/l)
α-lactalbumin (0.6-1.7 g/l)
serum albumin (0.2-0.4 g/l)
immunoglobulins (0.5-1.8 g/l)

Figure 2-1. Whey protein distribution in cheese whey (from Eigel et. al.,1984).

2.1.1. β-lactoglobulin

In cow's milk β-lg is the most abundant whey protein. Six genetic variants are already known (Eigel et al., 1984). The variants A and B are dominant and occur in nearly equal frequencies. The primary structure of each variant was determined (Swaisgood, 1982). The β-lg monomer with molecular weight (M.W.) of 18,400 daltons (D) is built from 162 amino acids. It contains two disulphide bridges (cystine) and one free thiol group (cystein). A variation in amino acid composition between the genetic variants is shown in Table 2-1. In the pH range 3.5-7.5 the protein exists as a dimer. However, between pH 3.5 and 5.2 dimers of both variants A and B may associate to form octamers. Association of β-lg A is much stronger compared to variant B. The structure of crystalline bovine β-lg has been determined in detail only recently (Papiz et al., 1986). From that work it had been concluded that, as a globular protein, the β-lg contains about 50-60% antiparallel β-sheet and 15-20% α-helix; the remaining 15-20% represent amino acid residue in a random non-repetitive arrangement without a well defined structure.

The protein shows remarkable stability at a low pH (Kella and Kinsella, 1989) resisting acid-induced denaturation at pH 2.5. However, β-lg denatures at an alkaline pH (McKenzie, 1971). At intermediate pH values the β-lg aggregates and undergoes conformational changes some of which have been detected by spectroscopic techniques (Swaisgood, 1982). At the pH 7.5 the structure of β-lg monomer is reversibly altered (Tanford et al., 1959). The

Table 2-1. Variations in amino acid composition of genetic variants of β-lg (Eigel et al.,1984).

Position	variant A	B	C	D
45	Glu	Glu	Glu	Gln
59	Gln	Gln	His	Gln
64	Asp	Gly	Gly	Gly
118	Val	Ala	Ala	Ala

transition is characterized by the release of a buried carboxyl group, the increase in the reactivity of a free sulfhydryl group and the change in environment of a tyrosine residue. The hydrogen binding equilibria and the pH titration curve for β-lg were studied initially by Cannan et al. (1942). Basch and Timasheff (1967) provided additional details for individual genetic variants. The maximum acid-binding capacity is 40 cation groups per dimer and total number of anionic groups is 50 in variant A and 52 in variant B. The variants C and D have 40 cationic groups and 50 anionic carboxyl groups per dimer (Basch and Timasheff, 1967; Brignon et al., 1969). The isoelectric point of this protein was identified as the pH 5.3-5.4 (McKenzie, 1971).

Above its isoelectric pH the β-lg may be capable to bind calcium to some extent as claimed by Zittle et al. (1957). Carr (1956) measured binding potential for alkali metals and sodium binding was reported by Baker and Saroff (1965). More recently Baumy and Brule (1988) showed, that β-lg is capable to bind bivalent cations such as Ca, Mg, Mn, Cu, Fe and Zn. However, the information about the binding potential of β-lg is sketchy, especially with respect to the Ca binding and its effects in processing.

2.1.2. α-lactalbumin

The α-la is a compact globular protein with molecular weight of 14,200 D. The monomeric polypeptide chain consists of 123 amino acids. The protein exists in two genetic variants A and B. The variant B differs from α-la A by a substitution of arginine for glutamine at the position 10. High tryptophan and aspartate levels, the presence of single arginine and methionine residues, four intra-chain disulphide bridges and the absence of phosphoryl and sulphydryl groups are notable features of the primary structure of this protein. The revised amino acid sequence published only recently by Shewale et al. (1984) revealed five aspartate residues within region 79-88 suggested as a Ca binding site (Stuart et al., 1986).

The striking similarity of α-la with lysozyme lead to a suggestion that both proteins have an identical three-dimensional structure (Brew et al., 1967). The crystallographic study of Krigbaum and Kriegler (1970) and Stuart et al. (1986) indicated that α-la consists of about 20% α-helix and 15% β-sheet. Remaining 65% of the protein chain has unordered structure.

When it was found, that 1 mol of α-la is capable of binding 1-2 moles of Ca (Hiraoka et al., 1980), there has been thorough investigation of the protein-metal binding (Bratcher and Kronman, 1984; Murakami et al., 1982). The indication of conformational changes related to the α-la interactions with metals was postulated by Kronman et al. (1981).

The association constant for Ca in model systems has been determined to be in the range of 10^8-10^9 M^{-1}(Hamano et al., 1986; Permyakov et al., 1982; Murakami et al., 1982; Kronman et al., 1981). The pH range of isoelectric point is between 4.2-4.5 (McKenzie, 1971). The determination of hydrogen binding and titration curve of α-la is complicated by a structural transition (Kronman et al., 1964). The low accuracy of the isoelectric point determination is caused by the low solubility of the protein in isoelectric region as well as strong tendency to bind anions and to undergo an association reaction (Kronman and Andreotti, 1964). The loss of tightly bound Ca appears to be responsible for the conformational change of α-la around pH 4 (Kronman et al., 1981). This acid transition occurs as a reversible denaturation (Bernal and Jelen, 1984) followed by a slow aggregation process (Kronman et al., 1964).

2.1.3. Other whey proteins

The minor whey protein constituents, serum albumin and immunoglobulins, account for up to 25% of the total protein in the whey (Farell, 1988).

Bovine serum albumin is identical to blood serum albumin (Eigel et al., 1984). The protein represents the longest simple polypeptide chain (M.W. 66,300 D) of all the whey proteins. It consists of 582 amino acid residues with 17 intra-molecular disulphide bonds and one free thiol group per molecule. The SA shows a heterogeneous electrophoretic behaviour that might be ascribed to adsorption of fatty acids because the protein is a well known transport carrier for insoluble fatty acids in the blood circulatory system (Spector, 1975). The binding of fatty acids to bovine SA appears to be one of the important factors in stabilization of its structure (Bernal and Jelen, 1985). On acidification, the SA molecule undergoes acid denaturation which is ascribed to the mutual repulsion of positively charged amino acid residues (Haurowitz, 1963).

Immunoglobulins are a complex of large glycoproteins which possess antibody activity (Walstra and Jenness, 1984). They are present in milk and/or whey in low concentration but are found in collostrum in much higher levels. The chemistry of milk immunoglobulins has been reviewed by Butler (1969) and Butler and Maxwell (1972). Four distinct classes occur in milk, IgM, IgA, IgE and IgG (Eigel et al., 1984), the latter class subdivided to IgG_1 and IgG_2, consisting up to 80% of the immunoglobulins. As a group, the immunoglobulins are either monomers or polymers of a four-chain molecule consisting of two light peptide chains (M.W. 20 kD) and two heavy chains (M.W. 50-70 kD) joined by disulphide bridges (Walstra and Jenness, 1984). They are among the most heat-sensitive of the whey proteins (de Wit and Klarenbeek, 1984).

2.2. Ionic equilibria in whey

The principal ionic components of whey are Ca, Mg, Na, K, phosphate, citrate and chloride (Walstra and Jenness, 1984). To this list it is appropriate to add the lactic acid as a product of bacterial fermentation of lactose. The salt molecules and ions are in dynamic equilibrium and any change in chemical environment results in new retained equilibrium. Furthermore, these ions interact to form soluble complexes with each other and with whey proteins. The nature and extent of equilibrium interactions between salts alone and salts and proteins in a milk diffusate are fairly well known (Holt, 1985; Walstra and Jenness,1984; Brule and Fauquant, 1981; Pyne, 1962) and various prediction models exist (Wood et al., 1981; Lyster, 1981; Holt et al; 1981). However, no detailed quantifications have been published based on physico-chemical measurements of the acid dissociation constants and the association constants of the salt complexes occuring under various conditions in different whey systems.

The compositional data of individual components forming the ionic environment of various wheys were shown in Table 1-1. Chlorides are almost completely present as ions regardless of the whey type (Pyne, 1962). That is also true for monovalent cations Na and K (Holt et. al., 1981). Concentration of other ionic species can be roughly calculated from known dissociation constants (Table 2-2), but very few of these can be verified experimentally. The amount of phosphate in sweet wheys differs profoundly from that of acid wheys (Hill, 1986). In acid whey the phosphate is entirely present in a soluble monovalent form (Merin, 1979). The citrate concentration in milk serum independent of pH (Boulet and Marien, 1960) is identical to citrate levels found in sweet and acid wheys. However, Walstra and Jenness (1984) pointed out the possibility of citrate breakdown by lactic bacteria. Lactic acid exists in the whey mainly as a lactate and not as a free acid since its pK is 3.9 (Pyne, 1962).

Table 2-2. Dissociation constants of important acids present in milk (from Walstra and Jenness, 1984; Pyne, 1962).

	pK_1	pK_2	pK_3
phosphoric acid	2.1	6.9	12.0
citric acid	3.0	4.5	5.9
lactic acid	3.9	-	-

Lactic acid as a product of bacterial fermentation is not found in acid wheys obtained by direct acidification.

Smeets (1955) in his calculations based on dissociation constants of citric and phosphoric acids suggested that about 35% soluble calcium and magnesium are present as ions, 55% bound to citrate and about 10% bound to phosphate. For sweet wheys in a limited pH range, ion concentrations of individual components may be estimated from ion equilibria computed for a milk diffusate. The levels of 2.5 mM Ca^{2+} (Demott, 1968) and 0.8 mM Mg^{2+} (Holt et al., 1981) determined in milk diffusate and rennet whey are in general aggreement with calculated values (Holt et al., 1981). Compared to sweet wheys, calcium level in acid types of wheys is markedly higher. During acidification, the majority of colloidal calcium phosphate (CCP) of the milk is released from casein micelles and transported into the whey. As the dissociation of most acids present in the whey diminishes at lower pH, the phosphate and citrate complexes are becoming less stable and the Ca^{2+} activity increases. The total Ca^{2+} concentration in the acid whey at pH 4.6 calculated from data of Wood et al. (1981) is about 19 mM. Small proportions of cations in sweet whey, particularly Ca, are associated as counterions with whey proteins. Net positive charge of whey proteins in acid wheys may attract some anionic species.

The complexity and variability of salt distribution in wheys led many reseachers to the adoption of a "model system concept". Jenness and Koops

(1962) described the composition of salt solutions simulating milk ultrafiltrate designed for the study of milk protein heat stability. The application of dynamic model systems using ultrafiltration permeates in investigation of whey protein precipitation characteristics was reported by de Rham and Chanton (1984). In an extension of this, they proposed a mathematical model describing immediate mineral composition of whey UF permeate and retentate during various stages of industrial ultrafiltration (de Rham and Chanton, 1986). The mathematical description utilized analytical data determined under a variety of conditions. In a study of effects of whey ionic environment on whey protein functionality, Jelen and Buchheim (1984) used experimental model solutions prepared from acid and sweet whey UF permeates.

2.2.1. Complexation effects

The major cations present in whey or milk form anionic complexes with acidic species. A considerable part of Ca and Mg may be bound in soluble citrate complexes (Jenness and Patton, 1959). Analogues of such complex ions may be also formed with phosphates especially at pH > 7 (de Rham and Chanton, 1984).

The coordination compounds or complex ions are formed from metal ions acting as electron pair acceptors with electron donors. The complex-forming ability of individual metals is derived from their electron configuration (Schwarzenbach, 1952a). The donor species or ligands such as carboxyl, amino, hydroxyl and many other similar types of groups must have at least one pair of unshared electrons. The complex formation is enhanced further by electrostatic attraction existing between metal ions and anionic ligands. Additional factors affecting complexation are metal electronegativity, ionic dimension, ionic charge and spatial configuration. As derived from the law of mass action the equilibrium constant of complex-forming reaction is a criterium used to evaluate the complex stability. Its value determined under defined conditions will change in the system of different physico-chemical characteristics. The stabilities of metal-ion complexes increase at higher pH as ligands become more negatively charged.

Organic compounds containing a carboxyl group are considered to be powerfull sequestrants. The ability of -COO$^-$ group to function as a ligand for a metal ion can be assessed from the behaviour of the acetate ion. Acetate complexes are not particularly stable but they are formed by practically all cations with multiple charge (Schwarzenbach, 1952b). The complexing power of a carboxyl group is enhanced by the presence of another coordinating group in the ligand such as hydroxyl or amino. In this case, the metal ions are bound exclusively in the more stable form of the chelate ring. Therefore, the complex-forming ability of citric and lactic acids (both hydroxycarboxylic acids present in cheese whey) is higher compared to monocarboxylic acids.

Complexes of lactic acid (2-hydroxypropanoic acid) with variety of metal ions such Mo, Cu, Co and lanthanides are known and their stability constants (K_{ML}) have been determined. The general trend is a bidentate binding of lactate via both the hydroxyl and carboxyl groups. However, there is only limited complex formation between lactate ligands and major cations occurring in whey. Lactic acid does not appear to form complexes with monovalent ions such as K and Na. Based on Schwarzenbach's classification, alkali metal cations have only a slight tendency to form complexes. Furthermore, thermodynamic stability of such complexes is low and the equilibrium is shifted towards dissociation of those complexes. The values of stability constants of Ca (log K_{ML} [MLac] = 0.9) and Mg (log K_{ML} [MLac] = 0.73) lactate complexes suggest also low stability of these compounds (Sillen and Martell, 1971).

The coordination chemistry of citric acid (2-hydroxy -1,2,3 - propane tricarboxylic acid) has attracted attention in the past and significant progress has been made in understanding the structures of its complexes. Citric acid has a great tendency to coordinate to the metal ion through one terminal carboxyl group and the central carboxyl group as a bidentate ligand forming five-membered chelate ring, preferably with di- and trivalent cations (Wilkinson, 1987). Similarly to lactic acid, complexes with Na and K have almost no significance (log K_{ML} [MCit] = 0.70 and 0.59 respectively). The formation constants of magnesium and calcium complexes indicate much higher stability of these complexes (Table 2-3). However, the investigation of

Na and K complexation by citrate ligands showed the cation of the ionic medium can influence the ionization equilibria and should be taken into account for this effect on the dissociation of citric acid (Pearce, 1980). In addition, a ligand can be protonated with consequent decrease of chelating power. Bjerrum (1950) has shown that there is a general relationship between dissociation of the hydrogen ion and complex formation. A similar relationship must be expected to exist with complexes between the dissociation constant of the last proton of the complex forming anion (Table 2-2) and the stability constant. It should be also noted that the stability constants are only valid for the specified medium with ionic strength of 0.1. In solutions of ionic strength appreciably less than 0.1 the stability constant will be somewhat larger than the values quoted above, whereas in solution with ionic strength more than 0.1 the complexes are correspondingly weaker.

The phosphoric acid as a part of a native whey system participates also in complexing of whey metal cations. However, its complexing ability is decreasing profoundly at lower pH (Jenness and Patton, 1959). Not much information is available on stability of these complexes in whey. Phosphates are active sequestrants above pH 7, which is usually out of the range of most whey processing situations.

Chelators are being employed in various studies of metal-protein equilibria in a whey system (Kronman et al., 1981; Bernal and Jelen, 1984; Hiraoka et al., 1980; de Wit et al., 1983). The most frequently used sequestrant

Table 2-3. Formation constants for Ca and Mg citrate complexes (from Pearce, 1980).

metal ion	log K_{ML} (MH_2Cit)	log K_{ML}($MHCit$)	log K_{ML}($MCit$)
Mg	0.6	1.78	3.63
Ca	1.0	2.03	3.64

is ethylenediaminetetraacetic acid (EDTA), mainly as its disodium salt. EDTA with the general formula H_4Y is a weak acid, for which $pK_1=2$, $pK_2=2.67$, $pK_3=6.16$ and $pK_4=10.26$. These values indicate that the first two protons are lost much more readily than the remaining two. In addition to four acidic hydrogens, there are two nitrogen atoms having unshared pair of electrons; the molecule thus has six potential sites for bonding with metal ions and may be considered to be a hexadentate ligand. It is apparent that in moderately acid media (pH 3-6) H_2Y^{2-} is the predominant species; in the region of pH 6 to 10, HY^{3-} is the major constituent. Reaction performed in weekly acidic solutions are best written as

$$M^{n+} + H_2Y^{2-} === MY^{n-4} + 2H^+$$

Hydrogen ions are liberated during the formation of metal complex. Therefore, the lower pH will decrease the equilibrium of complex formation. The decrease of chelating power may be obtained by calculation of a conditional formation constant from a corresponding stability constant and the pK values of a complex-forming ligand. In strongly acidic solutions complexes of aminopolycarboxylic acids such as EDTA are able to take up a proton

$$MZ^{n-4} === MHZ^{n-3}$$

The extra proton appears to be attached to a carboxylate ion that is no longer used as a ligand. Hence the stability of the complex at low pH (< 3) is somewhat greater than that calculated as above.

The stability constants (log K_{ML}) of divalent cations are reported as 10.7 for calcium and 8.67 for magnesium (Sillen and Martell, 1971). Because of the influence of pH the complete complexation (100%) of Mg with a maximum found at pH 9 will become less significant (10%) at pH 5 (Wilkinson, 1987). For calcium with its higher stability constant this decrease is less dramatic. In simple buffer systems below pH 3, Ca-EDTA complexes should be formed only to a limited extent (Pribil, 1972). This situation in a more complex system such as whey is much less clear and no experimental information exists.

2.3. Membrane separation of whey proteins

The aim of the membrane processing of cheese whey in general and of ultrafiltration in particular is to recover whey proteins in a more pure and undenatured form. The ultrafiltration is a separation technique, driven by pressure gradient, in which the membrane fractionates components of the liquid as a function of their solvated size and structure. In an ideal case an ultrafiltration membrane should be permeable to dispersed whey constituents of low molecular weight such as lactose and salts, while whey proteins are completely retained and concentrated relative to the other solutes in the retentate (Fig. 2-2). The rather high retention of some minerals, mainly Ca, Mg and P often found during ultrafiltration of cheese whey could be attributed to the appearance of a part of those constituents in a colloidal form or as complex-bound to whey proteins (Brule et al., 1974; de Wit and de Boer, 1975).

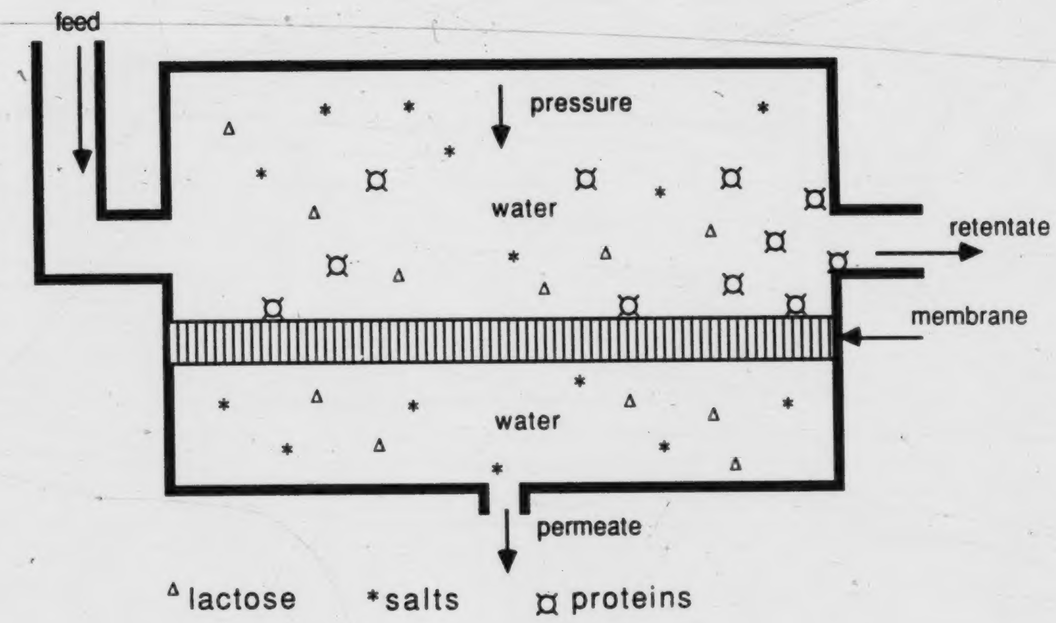
feed
pressure
water
retentate
membrane
water
permeate
Δ lactose
* salts
¤ proteins

Figure 2-2. A schematic representation of cheese whey ultrafiltration.

The negative and positive retention of inorganic constituents is influenced further by the "Donnan binding" effect (Hiddink et al., 1978). The major limitations in the application of membrane technology to whey processing are membrane fouling, low flux and high cost (Cheryan, 1986).

2.3.1. Membrane fouling

Fouling of a membrane can be described as a condition in which a membrane undergoes plugging or coating by some components in the feed stream in such a way that its output (flux) is reduced (Eykamp, 1978). The decrease in ultrafiltration rate caused by fouling must be clearly differentiated from the flux decline due to concentration polarization and membrane compaction. The phenomenon of concentration polarization is characterized by increasing hydrodynamic resistance close to the membrane surface. It can be reduced by changing operating parameters. In contrast, the fouling effects are irreversible (Michaels, 1968). An additional factor contributing to reduction of permeate flux is membrane compaction. Tarnawski and Jelen (1986) showed that the flux-suppressing effects of compaction and fouling are of the same magnitude at the transmembrane pressure ranging from 0 to 4 bars; above 5 bars, the compaction may markedly exceed fouling effects.

Lim et al. (1971) found that during concentration of cottage cheese whey by reverse osmosis, the permeation rate decreased with time because of an accumulation of foulant on the membrane surface. This material was predominantly protein including mainly casein, but also α-la and β-lg. Hayes et al. (1974) characterized casein associated with membrane fouling during ultrafiltration of whey as similar to κ-casein and referred to it as i-s casein (casein soluble at isoelectric pH of 4.6). Lee and Merson (1976a) reported that membrane fouling can be caused by several factors, including inorganic ions (P and Ca) which may be deposited on the membrane. For initiation of the deposit, charged groups or hydrophilic groups on a membrane have been indicated as nucleation sites capable of chemical interactions with certain solutes such as ions and proteins. As observed by Hickey and Hill (1980) the macropeptides derived from κ-casein contributed mainly to membrane fouling during ultrafiltration of Cheddar cheese whey. Tong et al. (1989) examined the membrane foulants isolated during Cheddar cheese whey ultrafiltration and found the α-la and several peptides generated by casein proteolysis adsorbed preferentially into the surfaces of membrane; however, the amount of β-lg found in an isolated foulant was also substantial.

Several studies indicated the strong influence of whey salts on the membrane fouling (Matthews et al., 1978; Hickey et al., 1980). Merin (1979) showed by X-ray analysis of membrane fouled with cottage cheese whey, that salts adsorbed quite strongly to the membrane and that the adsorption was enhanced by increase of the whey pH from 3 to 7. Higher pH in acid and sweet wheys also promoted sedimentation and deposition of insoluble Ca phosphates on the membrane leading to the fouling (Hayes et al., 1974; Kuo and Cheryan, 1983).

In a comprehensive review on this topic, Cheryan (1986) concluded, that although proteins are the major foulant of UF membranes, the characteristics of membrane fouling are determined unequivocally by simultaneous effects of pH, ionic strength, the nature of the salts present and other environmental factors.

2.3.2. Whey pretreatment for membrane processing

Alteration or control of the chemical and physical characteristics of the various types of whey has been shown to have a considerable influence on the operating characteristics of membrane processes. Flux rates during UF of cheese whey were shown (Harper, 1980) to be improved by prefiltration and centrifuging. Prefiltration with filter paper and with series of membranes of decreasing molecular weight cut-off progressively improved UF rate of cottage cheese whey (Lee and Merson, 1976b). Centrifugation, sedimentation and/or filtration can remove some of the larger fat globules and thus improve ultrafiltration performance (de Wit et al., 1983) and alter the final functional behaviour of the whey protein concentrates obtained by the membrane separation (Lim et al., 1971). Delaney and Donnelly (1977) suggested minimum pretreatment of cheese whey by centrifugal separation to obtain 0.05% residual fat and casein fines content. In addition, utilization of precipitation agents to remove lipids has been shown to have highly beneficial effect on the ultrafiltration rate (Attebery, 1971).

The preliminary observations on the effects of pH and calcium content in relation to whey heating before ultrafiltration led Hayes et al. (1974) to study this aspects in more detail. They found that, with HCl casein whey, heating to 80°C/15 s and adjustment of pH resulted in minimum fouling at a pH optimum in the region of 5.9. This treatment at least doubled flux rates as compared with those of the pasteurized whey at the normal pH of about 4.4. Cheddar cheese whey at its natural pH of 6 or above when heated at 85°C/15 s gave a flux at least 50% above that of pasteurized whey. They ascribed the reduction of fouling mainly to the aggregation of β-lg complex with casein-like components formed upon heating.

Lee and Merson (1976a) demonstrated that the flux improvement during ultrafiltration of cottage cheese whey can be achieved either by lowering pH below 4 or by addition of a sequestrant (EDTA) to chelate Ca. Furthermore, addition of electrolytes such as $CaCl_2$ or NaCl resulting in increase of ionic strength enhanced the transport through the membrane. Kuo and Cheryan (1983) acidified cottage cheesse whey up to pH 2.5 and observed significant improvement in the rate of filtration compared to untreated whey. Hayes et al. (1974), using ion exchange, showed that cation exchange in HCl casein whey gave a noticeable improvement in flux, particularly outside the pH range of 4 to 5.

Cation exchange, Ca replacement with Na, and ultrafiltration of cheese whey at low pH has been applied commercially (Ennis et al., 1981; Kuipers and Meggle, 1975). Demineralization of acid whey up to 95% was shown to improve flux rates even more than did cation exchange - probably, in view of published findings (Hickey et al., 1980), as a result of the lower ionic strength or reduction in phosphate content.

As claimed by Cheryan (1986), pretreatment of the cheese whey is the most effective way to maximize ultrafiltration performance. The effects of pretreatments are dependent on the type of whey, ultrafiltration equipment, membrane type and operating conditions.

2.4. Thermal behaviour of whey proteins

The thermal behaviour and heat stability of whey proteins are major factors in determining their functionality in food systems. The heat induced protein alteration or denaturation, a phenomenon intimately linked with the structural stability of the native protein, results frequently in the reduction of whey protein solubility. The heat response of individual whey proteins and the character of the induced changes were extensively studied from many different viewpoints including milk, wheys and purified proteins. Comprehensive reviews on the topic of whey protein thermal denaturation and aggregation are available (Lyster, 1971; Fox, 1982; Hill, 1986; Mulvihill and Donovan, 1987).

The capability of whey proteins to coagulate upon heating has been utilized for manufacture of a denatured whey protein concentrate referred to as "traditional lactalbumin", which can be used to fortify some meat and bakery products (Jelen, 1979) and in many other uses. Heat enhanced reactivity of sulphydryl groups of β-lg, presumably responsible for undesirable flavor development (cooked flavor) in milk and dairy products, may result in whey protein interactions via disulphide linkages (Sawyer, 1969). This in turn affects the heat stability of whey systems and also impairs the rennet clotting properties of milk (Fox, 1982).

The heat stability of whey proteins is determined primarily by their amino acid sequence (de Wit, 1981) and secondly by environmental effects such as pH, temperature, concentration and presence of salts. Differential scanning calorimetry (DSC) frequently employed in studies of the protein thermal denaturation revealed differences in thermal responses of the individual whey proteins (Table 2-3).

2.4.1. Isolated β-lactoglobulin

Specific attention was focused on the thermal behaviour of β-lg which, as a dominant whey protein, tends to govern the behaviour of the total whey protein system. As indicated in most studies, an initial thermal transition of β-lg appears to take place in the temperature range 60-85°C. A second transition temperature of β-lg was noted between 130-150°C and this was attributed to unfolding of the β-lg structure (de Wit and Klarenbeek, 1981; 1984). The magnitude of reported denaturation temperatures (Td) depends somewhat on

Table 2-4. Thermodynamic parameters of whey protein heat denaturation.

Protein	Conditions	Denaturation temperature (°C)	Denaturation enthalpy (kJ/mol)	Ref.
β-lg	SMUF[a], pH 6-7	72.8	227	1
	SMUF, pH 3.5	81.9	n.a.[b]	5
	4.5	81.2	n.a.	5
	6.5	75.9	n.a.	5
	phosphate, pH 6.75	70.5	230	2
	phosphate, pH 6.0	78	311	4
	H_2O, pH 3.5	85/140	240	3
	H_2O, pH 6.8	65-75/125-140	150-140	3
α-la	SMUF, pH 6.7	65.2	318	1
	SMUF, pH 3.5	58.6	146	6
	4.5	61.5	319	6
	6.5	61.0	289	6
	phosphate, pH 6.0	62	253	4

[a] simulated milk ultrafiltrate (Koop and Jenness, 1962)
[b] not available

1 Ruegg et al., 1977
2 de Wit and Swinkels, 1980
3 de Wit and Klarenbeek, 1981
4 de Wit and Klarenbeek, 1984
5 Bernal and Jelen, 1985
6 Bernal and Jelen, 1984

the interpretation of the thermograms. Most authors considered Td as the temperature of maximum deflection and found this to depend on heating rate and environmental conditions.

Studies on the thermal behaviour of purified β-lg made evident that maximum stability occurs at or below the isoelectric point. Ruegg et al. (1977) found that pH dependent Td decreased from about 80°C at pH 6.5 to 74-75°C at pH 7.3. De Wit and Klarenbeek (1981; 1984) reported similar observations, finding transition temperatures of 82, 78 and 71°C at pH 3, 6 and 7.5, respectively and also observing decreasing apparent denaturation enthalpies of 16.6, 15.9 and 11.2 J/g. Bernal and Jelen (1985) confirmed these effects of pH on β-lg denaturation in the pH range from 2.5 to 6.5; at pH 3.5 the denaturation temperature for an isolated β-lg preparation was 81.9°C compared to 81.2°C and 75.9°C at pH 4.5 and 6.5, respectively. Harwalkar (1980a, 1980b) found that β-lg remained completely soluble at pH 2.5, precipitated completely from solution at pH 4.5 and also precipitated at pH 6.5. The ionic strength of the solution enhanced the extent of precipitation. The β-lg heated at pH 4.5 and 6.5 was considered completely denatured while heating at pH 2.5 produced two molecular species; one native-like compound, soluble at pH 4.5, was indistinguishable from the true native form. The other species was considered an irreversibly altered form of β-lg. Harwalkar (1980b) concluded that denaturation of β-lg leads to partial protein unfolding that increases progressively with severity of the heat treatment.

2.4.2. Isolated α-lactalbumin

Based on recent studies α-la can be considered as the least thermostable among whey proteins (Table 2-3). The protein was found to be more thermo-sensitive at pH 3.5 than at pH 4.5-6.5 and this increased thermo-sensitivity further appeared extremely dependent on the calcium-protein binding (Bernal and Jelen, 1985). After removal of calcium the Td of α-la decreased by approximately 20°C and this was accompanied by significant reduction of the enthalpy of denaturation. These observations conflict with the earlier findings of Larson and Rolleri (1955) that α-la is the most thermostable whey protein. In this classical work, the degree of denaturation of whey proteins in milk was assessed using electrophoresis. The order of the observed heat resistance of individual whey proteins was: Ig < SA < β-lg < α-la. However, the α-la is the only major whey protein that is capable of renaturation following heating; renaturation up to 90% has been observed in model solutions (Bernal and Jelen, 1984; de Wit and Klarenbeek, 1984) and this may explain the results reported by Larson and Rolleri (1955). However, denaturation of α-la appears to be irreversible in a complex whey system (Bernal and Jelen, 1984).

Lyster (1970) investigated the denaturation mechanism of α-la and β-lg in skimmilk using an immuno-diffusion method. Denaturation of α-la was described as a first order reaction and denaturation of β-lg followed second order kinetics with separate rate constants for individual Td between 68 - 90°C and 90-135°C. Whey protein heat denaturation kinetics determined in cheese whey was in close agreement with that determined in skimmilk (Hillier and Lyster, 1979). However, data obtained from kinetic analysis of the whey protein thermal denaturation reaction led Danneberg and Kessler (1988) to conclude, that denaturation of β-lg and α-la was best described with a reaction order of 1.5 and 1, respectively.

2.4.3. The effects of environmental conditions on whey protein thermostability

The complexity of environmental conditions in whey alters susceptibility of whey proteins to denaturation compared to defined model solutions. Guy et al. (1967) observed that the proteins in cottage cheese whey (acid type) became less heat stable as pH increased from 3.5 to 6.7. Observation of Hillier et al. (1979) indicated enhanced heat stability of α-la and β-lg in cheese whey at pH 4 compared to that in neutral and alkaline pH regions. The maximum heat instability of whey protein concentrates was observed at pH 5.5 with second labile region at pH 8-9 (Varunsatian et al., 1983). As reported by Bernal and Jelen (1985), a DSC revealed maximum heat resistance of whey protein concentrates in the low pH zone of 2.5-4.5. High resistance of whey protein to coagulation in heated whey below pH 3.5 was noted by Jelen and Buchheim (1984).

Harwalkar (1979) observed that the proteins in an acid whey remained soluble during heating (90^{o}C/20-30 min) at pH 2.5; in contrast, the majority of whey proteins precipitated on heating at pH 4.5 or 6.5. This led to a conclusion that the lack of heat induced precipitation at pH 2.5 compared to pH 4.5 or 6.5 was probably due to a greater net positive charge of the proteins and/or to the fact that little sulphydryl oxidation or sulphydryl-disulphide exchange would occur at such a low pH. In this system whey proteins appeared to be incompletely denatured by heating at pH 2.5. Similar observations of detrimental effects of increasing pH on whey protein thermal stability in rennet whey were reported by Donovan and Mulvihill (1987).

The markedly pH dependent whey protein aggregation is further influenced by the presence of divalent cations, particularly calcium. The heat precipitation characteristics of β-lg and α-la measured by Townend and Gyuricsek (1974) exhibited minima at pH 5.8 and 4.8 respectively. The added calcium induced substantial heat precipitation of β-lg above pH 6 but it did not affect the thermal stability of α-la over the entire 2-12 pH range. These workers also showed that the maximum heat induced protein precipitation in an acid whey occurred at pH 6.5. Nevertheless, they pointed out that the thermal response of the purified individual proteins cannot be directly extrapolated to their behaviour in "real" whey systems.

Zittle et al. (1957) demonstrated, that in the absence of calcium, maximum precipitation of β-lg occurs at its isolelectric point. They proposed a role for Ca and pH in the thermocoagulation of β-lg: precipitation of β-lg in heated, calcium-containing solutions depends on the net protein charge resulting from the binding of Ca^{2+}, and is analogous to isoelectric precipitation. In the 6-8 pH range the amount of calcium bound by β-lg parallels the protein negative charge. This hypothesis was confirmed by de Wit and Klarenbeek (1984). In their interpretation an increased thermal sensitivity of β-lg to Ca aggregation above pH 6.5 results from higher reactivity of thiols in the polypeptide chain at this pH. Kenkare et al. (1964) found that in acid whey containing a higher concentration of calcium phosphate than ultracentrifuged (UC) whey, the whey proteins were less stable to heat than those in UC whey. Roeper (1971) reported that after increasing the calcium content of Cheddar cheese whey, the whey flocculated during heating in a manner similar to acid whey. Morr and Josephson (1968) suggested that whey protein aggregation is a multireaction process, the third stage of which involves the formation of gross aggregates, sedimentable at 1000 x g, and this reaction is calcium-dependent.

Donovan and Mulvihill (1987) confirmed the dominant role of Ca^{2+} and its interrelationship with pH in whey protein aggregation. They observed that addition of Ca^{2+} had no effect on the extent of aggregation occuring in a heated rennet whey between pH 5-6 but, at pH values of 6.5 and 7.0, added Ca^{2+} promoted substantial increases in aggregation comparable to that occuring in an acid whey at the same pH. These results support the findings of de Rham and Chanton (1984) who proposed that insolubilization of whey proteins by heat treatment is affected primarily by their ionic environment; a protein solubility was impaired during heating in the pH range 4-6 but not above pH 6 unless available calcium exceeded a critical concentration. Similarly de Wit (1981) and de Wit and Klarenbeek (1984) demonstrated a high sensitivity of desalted whey systems to Ca^{2+} triggered protein aggregation compared to regular rennet whey, where citrate and phosphate in particular compete with whey proteins for calcium.

2.4.4. Heat induced whey protein interactions

Because of important technological implications, numerous studies have been focused on investigation and characterization of interactions between individual whey protein fractions and between whey proteins and caseins upon heating. Of the heat-induced interactions which occur in milk, the longest established and best studied is the complexation between β-lg and κ-casein (κ-cn). It is believed that intermolecular disulphide bonding is important in formation of this complex (Burton, 1984). However, the evidence of such mechanism is not equivocal. Morr (1965) noted that the κ–cn-β-lg complex observed in heated skimmilk had different properties than the complex formed upon heating purified κ-cn and β-lg mixtures. Sabarwal and Ganguli (1972) showed that micellar casein stabilizes whey proteins against denaturation and suggested that the stabilizing effect is due to the blocking of the free SH group of β-lg through complexation with κ-cn. The results and conclusions of Kenkare et al. (1964) and Morr and Josephson (1968) indicated that added individual caseins, whole caseinate or casein micelles do not stabilize whey proteins against denaturation, but prevent precipitation of denatured whey protein systems. It was concluded that this stabilizing effect is not a result of the disulphide interchange mechanism but the consequence of non-specific calcium-linked complex formations between caseins and whey proteins.

Until now, no evidence of any interactions between κ-casein and α-lactalbumin was reported. Baer et. al. (1976), using a serological technique, declared that α-la cannot directly form heat-induced complexes. It appears, that possible α-la-κ-cn complexation may occur indirectly via interaction of α-la with β-lg and this complex seems to interact with κ-cn. Such a mechanism was suggested by Elfagm and Wheelock (1977) and evidenced recently in a radiolabelled study carried out by Noh et al. (1989). Earlier, α-la and β-lg have already been reported to interact upon heating (Wiechen and Knoop, 1974; Elfagm and Wheelock, 1978). Nevertheless, the nature of this interaction has not been fully elucidated. Noh et al. (1989) using purified radiolabelled α-la and β-lg heated together did not observe any signs of α-la-β-lg complexation after separation of the protein-containing solution by gel filtration.

In a proposed model mechanism of whey protein thermal behaviour Hill (1988) assumed that thiol-disulphide interchange plays the principal role in protein aggregation. Upon heating, proteins in whey systems precipitate alone (β-lg) or may co-precipitate (α-la-β-lg). In addition, non-specific whey protein aggregation is partially attributed to hydrophobic interactions. In milk, whey proteins are completely denatured in 5 min at 90^{o}C (Fox, 1982); they remain in solution and may co-precipitate with casein by the addition of acid or soluble Ca salt (Southward and Aird, 1978). Compared with casein, co-precipitates give a 7-21% higher yield of protein, have a wider range of functional properties and a higher nutritional value.

Despite the research advances in the last few years, nearly half of the whey produced globally is still being wasted. Continued fundamental studies of the physico-chemical, thermal and nutritional characteristics of various whey protein systems coupled with vigorous technical marketing of the whey protein containing products will be needed to implement wider commercialization of whey processing technology. The desire of the dairy industry to increase its economic returns and the current scarcity of milk proteins will undoubtedly result in further whey processing research studies.

2.5. References

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3. RAPID CLARIFICATION OF COTTAGE CHEESE WHEY BY CENTRIFUGATION AND ITS CONTROL BY MEASUREMENTS OF ABSORBANCE[1]

3.1. Introduction

The utilization or effective disposal of cottage cheese whey continues to be one of the important problems of the dairy industry. Traditional whey processing techniques such as evaporation and drying are often too costly for the relatively small amounts of cottage cheese whey available at a given location. A more realistic alternative for the small processor is the direct use of whey in various types of drinks, spreads, yogurts, and other fermented dairy products (4, 6).

During draining, variable amounts of coagulated casein in the form of casein fines may be included in the whey. Bender and Tuckey (2) reported an average of 0.32% of casein fines in liquid whey causing cloudy appearance. For products such as whey based drinks, where good clarity and no subsequent sedimentation is desirable, the fines have to be removed. Whey clarification is also a necessary pretreatment step for many membrane processes (3).

Casein particles can be removed from whey by simple sedimentation. While large particles will settle quickly, the rate of sedimentation in the main clarification phase is slow. A much faster method of clarification commonly used in the dairy industry is centrifugation, but the effectiveness of the casein fines removal has to be monitored. A simple method for evaluation of the casein fines content in whey developed by Raab et al. (9) was based on determination of casein fines volume sedimented in calibrated centrifuge tubes. However, a more rapid and convenient method with higher accuracy was required for industrial quality control purposes (Sholdice, Palm Dairies Ltd., personal communication).

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[1] A version of this chapter has been published. G. Patocka and P. Jelen 1989. Milchwissenschaft 44 (8): 501-503

The objectives of this work were: (a) to quantify the effectiveness of centrifugal clarification for removal of residual casein from cottage cheese whey in comparison to gravitational sedimentation; (b) to develop a rapid, industrially applicable method for evaluation of the level of whey clarity needed in further processing; and (c) to show the effectiveness of centrifugal fines removal from cottage cheese whey for improvement of ultrafiltration flux.

3.2. Materials and Methods

Cottage cheese whey received from a local dairy plant was obtained immediately after draining from the cheese vat. Part of the whey was allowed to settle at 22 ± 1^{o}C for 24 h and a sedimentation profile was determined by monitoring absorbance by simple turbidimetric measurements using the EQA-1 instrument (Gaulin's Corp., Everett, Mass.), as described earlier (5). Each value was obtained as the average from three independent turbidimetric readings.

The remainder of each whey batch was centrifuged on a pilot plant continuous clarifying separator LAPX 202 (Alfa-Laval AB, Tumba, Sweden). At maximum speed of the separator (8,400 x g) the feed rate was controlled between 1500 ml/min and 250 ml/min to produce whey with various clarity levels, determined by absorbance measurements as above. The experiments were replicated with five different batches of cottage cheese whey.

To correlate the absorbance with casein fines contents, 30g samples of the clarified wheys were centrifuged in a laboratory centrifuge IEC HN-S II (Damon/IEC Div., Needham, Mass.) at 10,700 x g for 10 min. The supernatant was decanted and the sediment was filtered, washed 3x with distilled water, dried and weighed. The total protein content of the whey before centrifuging and in the centrifuged supernatant was determined by a routine Kjeldahl method (1).

The importance of centrifugal clarification as a pretreatment for maximization of ultrafiltration (UF) flux was illustrated by UF of wheys with various amounts of casein fines prepared by centrifugation on the LAPX 202 centrifuge to desired absorbance. The UF runs were carried out for 5 h on a DDS Lab-20 UF module (De Danske Sukkerfabriker, Nakskov, Denmark) with two sandwiches of GR-61 PP membranes (M.W. cut-off 20,000 D); filtration area was 0.072 m^2, transmembrane pressure 350 kPa. The methodology used for membrane pretreatment and cleaning was described earlier (8). All UF experiments were in duplicate.

3.3. Results and Discussion

The absorbance of freshly drained whey measured within the first 15 min was in the range of 0.95 to 1.17. Sedimentation profile for whey with the initial absorbance of 1.17 is shown in Fig. 3-1. In the initial period (2 - 3 h) the sedimentation proceeded fast and most of the large size particles settled, with the absorbance decreasing to the 0.85 - 0.95 range. The clarity reached after 24 h of sedimentation was characterized by absorbance in the 0.45 - 0.40 range. In contrast, when the same wheys were processed on the continuous desludging centrifuge at optimal conditions (the maximal centrifugal force 8,400 x g, feed rate 250 ml/min) the final absorbance was in the 0.06 - 0.07 range. The feed rate of 250 ml/min appeared to be the limiting input. Further lowering of the feed rate had no effect on the final absorbance of the clarified whey, while at higher feed rates, the clarification was less effective as shown by higher absorbance values.

The relationship between the absorbance and the amount of residual casein in the whey is summarized in Fig. 3-2. For all absorbance values higher than 0.90 the reproducibility of the correlation with the casein fines content was poor. The obvious reason appears to be the variable presence of either curd clusters or large size casein particles in the freshly drained whey. When most of the large particles were completely removed in the first period (2 - 3 h) of sedimentation, the substantial decline in the amount of residual casein fines to be removed by the centrifugation gave much more reproducible correlations (Table 3-1). For the absorbance values lower than 0.07, no casein fines were detected. Whey protein contents determined in the non-centrifuged whey samples and the supernatants from the laboratory centrifugation trials were in the range of 0.79 to 0.83 (% w/w), which is in good agreement with reported values for acid wheys (7). Thus any incidental removal of whey proteins by centrifugation of residual casein fines seems to be unlikely. The absorbance measurements were well reproducible (standard error ± 0.01) with respect to the determinations of residual casein, when the content of coagulated casein was less than 0.1%.

The dependence of UF flux on the efficient casein fines removal from cottage cheese whey is illustrated in Fig. 3-3. The flux for the centrifugally clarified whey (absorbance = 0.13) was about 100% in the initial period and almost 80% after 5 hours of operation above that of the fresh whey (absorbance = 1.01), and about 30% above the flux obtained with 24 h sedimented whey (absorbance = 0.45). Centrifuging the whey to the clarity level of 0.45 took about 1 hour, while less than 2 hours were needed at the low feed rate of 250 ml/min to reach the absorbance of 0.13. Higher clarification levels (i.e. absorbance below 0.4) cannot be reached by sedimentation in industrially realistic conditions.

3.4. Conclusions

The monitoring of absorbance in cottage cheese whey is a simple, rapid and reproducible method for evaluation of the effectiveness of casein fines removal. The method is suitable for routine industrial use, as shown by its recent application in process development conditions of a large dairy manufacturer. To reach the required clarity levels, whey centrifugation is much more effective than sedimentation. Complete removal of casein fines by gravity sedimentation is not possible within industrially acceptable process time. Complete whey clarification by centrifugation can significantly improve ultrafiltration rates.

3.5. Tables and Figures

Table 3-1. The amount of casein fines and the corresponding absorbance index values for cottage cheese whey.

Absorbance Index[a]		Casein Fines Content[b]	
X	SD($\times 10^{-3}$)	X	SD($\times 10^{-3}$)
		-----% (w/w) -----	
0.80	5.4	0.1100	4.20
0.69	4.1	0.0392	1.28
0.60	6.6	0.0301	1.21
0.45	7.6	0.0215	0.59
0.34	2.1	0.0086	0.31
0.28	8.2	0.0058	0.34
0.15	3.2	0.0038	0.12
0.12	5.1	0.0014	0.06

[a] Means and st. dev. of three replicate readings.

[b] Means and st. dev. of three replicates of the same sample.

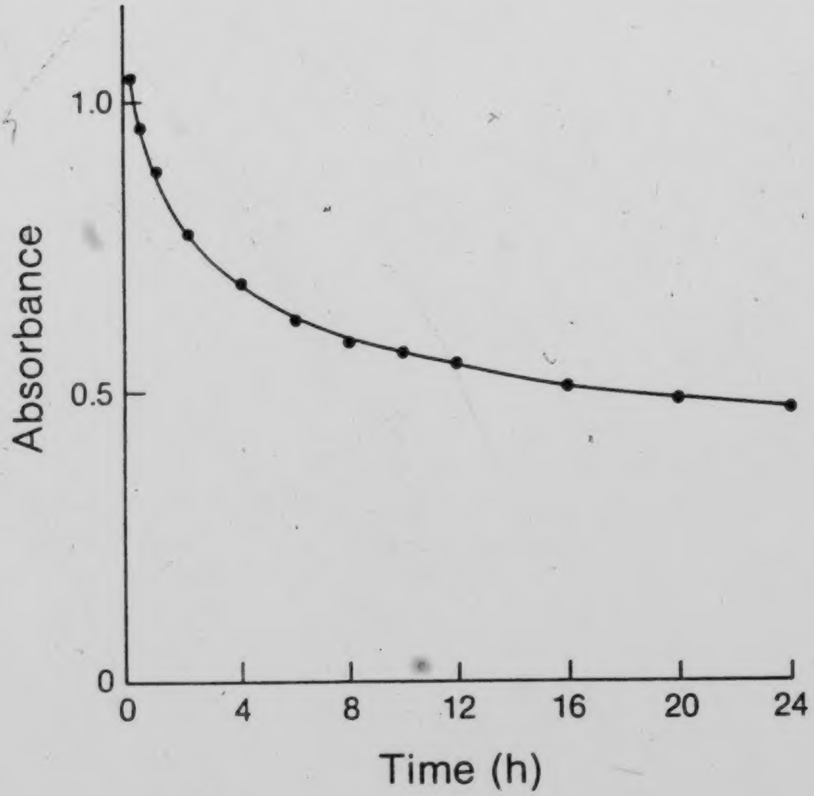

Figure 3-1. Sedimentation profile for casein fines in cottage cheese whey.

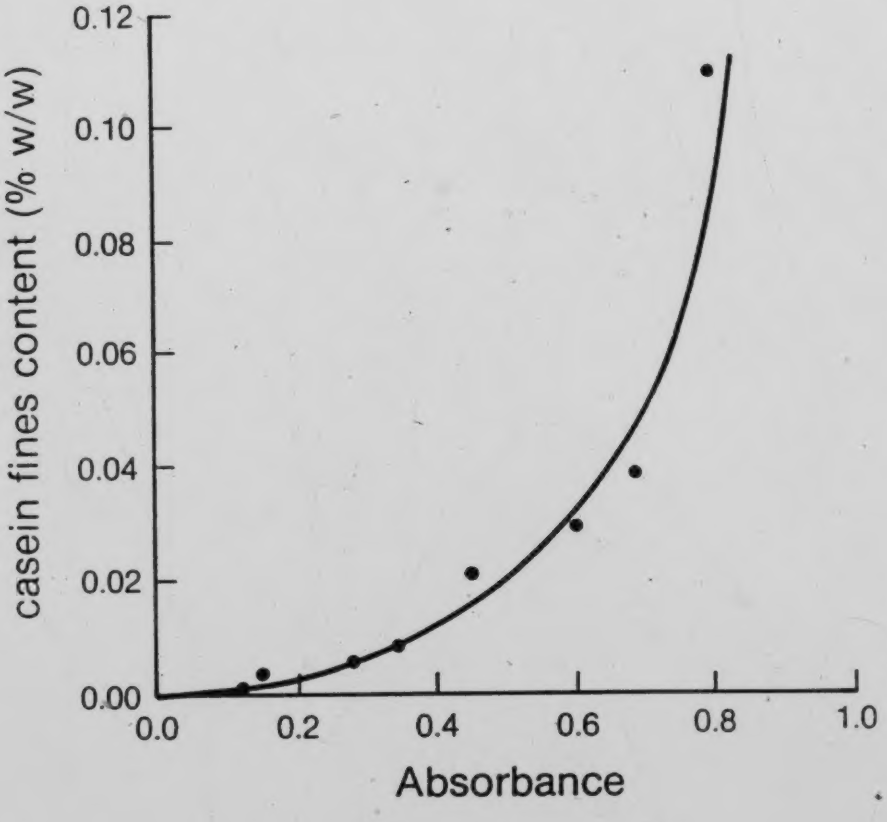

Figure 3-2. Relationship between the casein fines content and absorbance at 900 nm for cottage cheese whey (datapoints are averages of three replicate A.I. readings and casein fines determinations for each whey sample; average st. dev. for casein fines content was 1.006×10^{-3} % w/w; the 3rd degree polynomial correlation coefficient R = 0.98).

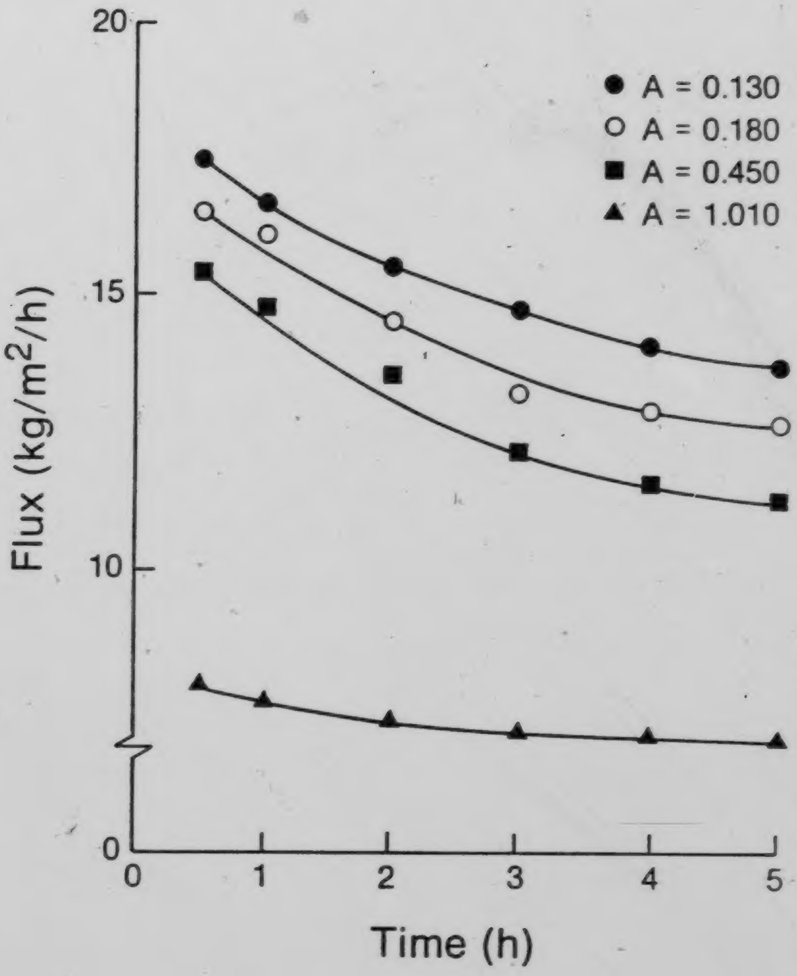

Figure 3-3. Ultrafiltration flux of cottage cheese whey with various clarity levels filtered on DDS Lab-20 module.

3.6. References

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4. PROTEIN COAGULATION IN SWEET AND ACID WHEYS UPON HEATING IN HIGHLY ACIDIC CONDITIONS[1]

4.1. Introduction

The loss of whey protein solubility upon heat treatment could result in serious quality defects in whey drinks and other food products based on liquid cheese whey. Recent reports (2, 8, 10) indicated that stability of whey proteins against heat-induced precipitation increases significantly in a rather narrowly defined pH range of 3.9-3.8. Many whey drinks currently available on West German and other European markets are stabilized at this very pH range to optimize sensory acceptability (Jelen, unpublished data). To minimize the frequently observed problem of sedimentation in these products, it is important to increase our understanding of the specific mechanism leading to higher heat stability of whey proteins in the various cheese whey systems that may be used in the manufacture of whey drinks.

One of the important factors affecting behaviour of whey proteins upon heating is the amount and the state of calcium present in the system (4, 5, 16). Numerous studies on Ca^{2+} binding to isolated whey proteins were reviewed recently by Hill (8) illustrating the role of Ca^{2+} in stabilization of both α-lactalbumin (α-la) and β-lactoglobulin (β-lg) against heat precipitation. The ability of β-lg to bind calcium was first shown by Zittle et al. (17), while Hiraoka et al. (9) demonstrated that α-la is a calcium metallo-protein. The presence of [illegible] Ca^{2+} binding sites for bovine α-la was established by Kronman et al. (11). Bernal and Jelen (3) illustrated the importance of Ca^{2+} for stabilization of α-la against heat denaturation at pH 4.5 or higher; unavailability of calcium resulted in lowering of the denaturation temperature from 61°C to 40.8°C, accompanied by the loss of the reversibility of the reaction.

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[1] A version of this chapter has been published. G. Patocka, A. Renz-Schauen and P. Jelen 1986. Milchwissenschaft 41(8): 490-494

Behaviour of isolated whey protein species (especially α-la and β-lg) in the highly acidic range below pH 4.0 has been studied mainly with model systems. As indicated in Hill's comprehensive review of this topic (8) both α-la and β-lg undergo structural changes below pH 4.0 leading to thermal behaviour which may be different for that at pH above 4.0. In particular, the dimer configuration of β-lg dissociates at pH below 3.5; due to electrostatic repulsion, the resultant monomer form is resistant to coagulation upon heating (5, 6, 7). On the contrary, the conformational change of α-la at pH below 4.0 is accompanied by the release of the bound calcium which may lead to acid-induced denaturation and aggregation (3, 11, 12). The effects of ionic strength, specific metal ions, temperature, pH and other experimentally controlled parameters appear interrelated and dependent on composition of these model systems.

Much less information is available on protein precipitation effects in natural whey systems, particularly with respect to differences in mineral composition of sweet (rennet) and acid wheys. Modler and Emmons (15) proposed a method for recovery of whey protein concentrate for sweet whey by precipitation at pH 4.5 after heating at the 2.5-3.5 pH range at which some heat denaturation (but no coagulation) was induced. Our previous report (10) dealt with heat precipitation stability below pH 4.0 of the total whey protein fraction in laboratory prepared acid wheys. In continuation of these studies, the present objectives were 1) to compare the stability of whey protein against visible heat precipitation at pH below 4.0 in laboratory prepared and industrial whey systems, and 2) to study the effects of calcium content and calcium availability on the whey protein stability upon heating of these complex whey systems at acidic conditions.

4.2. Materials and Methods

The general methodology of laboratory whey preparation, subsequent heat treatment, and determination of heat stability was previously reported (10). Because of the wider scope of the present studies, minor modifications and/or additional procedures were adopted.

Both acid and sweet laboratory wheys were prepared from pasteurized skimmilk obtained from a local dairy processor using concentrated (85% v/v) lactic acid or single-strength rennet (0.2 ml/1000 ml milk), respectively. Laboratory wheys were recovered by filtering the clotted milk through a filter paper and were left standing overnight to sediment casein fines. Industrial wheys were obtained from local cheese manufacturers. Heating experiments were carried out in 40 ml quantities using 100 ml volumetric cylinders. Samples were heated in water bath to 92^{o}C . Time for reaching this temperature varied from 6 1/2 to 7 minutes, and was not included in the total time of heating reported below. After heating for defined time periods the samples were immediately cooled in an ice water bath and, after temperization of each sample to 20±0.5^{o}C, turbidimetric measurements of absorbance index (AI) were performed on an EQA-1 instrument - a fixed 900 nm wavelength spectrophotometer (Gaulin's Corp., Everett, Mass.). The AI range of the instrument (0.000 to 1.999) was found satisfactory for this determination (10). It should be emphasized that this AI range represents a relatively minor difference in optical appearance of the sample.

The pH adjustments of the samples before heating were made with concentrated lactic acid (85% v/v, Fisher Sci.). The pH was measured on an Orion model 601A pH meter (Orion Research Inc., Cambridge, Mass.). Where required by the experimental design, calcium was chelated by disodium salt of ethylenediamine tetraacetate (EDTA) or by citric acid monohydrate using various chemical equivalent ratios calculated from the calcium content of the wheys determined previously. Calcium replacement with sodium was achieved by passing the whey through an ion exchange column (downflow) filled up with Bio-Rex 70 resin in the sodium form (BioRad Laboratories, Richmond, CA). After each pass through the column, the resultant pH (8.0 and 9.3 for the acid and sweet wheys, respectively) was adjusted with lactic acid to obtain values desired for the heating experiments.

Content of calcium and phosphorus were determined by atomic absorption with AtomScan spectrophotometer (Jarrel-Ash Div., Fisher Scientific

Co., Waltham, Mass.), while other analytical data (protein, total solids) were obtained using routine analytical methods (1). All heating experiments and all turbidimetric measurements were repeated at least twice, and the entire experimental block was replicated as indicated using at least two different whey supplies for each case. The variability of the replicate turbidimetry data was minimal; some differences due to the initial turbidities of the wheys used did not affect the general trend observed.

4.3. Results and Discussion

In several series of experiments, stability against heat induced coagulation was compared between laboratory-prepared sweet and acid wheys. The average results are summarized in Fig. 4-1. While the results for the acid wheys (Fig. 4-1a) are in good agreement with previously reported data (10), the sweet wheys (Fig. 4-1b) showed a slight but distinct decrease in heat stability. Increase in turbidity upon heating at 92°C was higher than in acid wheys of corresponding pH and the critical coagulation pH decreased. (The "critical pH range" was taken arbitrarily as that 0.1 pH unit interval within which a noticeable transition in heat precipitation behaviour and/or visible flocculation occured during 20 min heating at 92°C). In our interpretation, lower heat stability is indicated by lower pH needed to eliminate flocculation or minimize increases in turbulence upon heating; the farther this pH from the general transitory region of pH 3.8-3.9, the lower the heat stability of the given system. In general, the magnitude of the heat stability differences between sweet and acid whey systems was small. At pH 3.5 or lower both systems showed complete stability against heat-induced coagulation as evidenced by constant AI readings throughout the whole 20 min heating period. The average calcium and phosphorus contents of all experimental wheys used in this work are summarized in Table 4-1 together with the critical pH values observed. It can be seen that in general, lower heat stability corresponds to lower calcium content.

To further test this postulated relationship, variable amounts of EDTA were added to the acid wheys before heating experiments. As shown in Fig. 4-2a, the heat stability of the protein was indeed reduced as the EDTA content in the whey increased. At the 1:1 EDTA/Ca^{2+} equivalent ratio, the heat stability of acid whey was reduced to approximately that of the sweet whey, and at the higher EDTA/Ca^{2+} ratio of 2:1 the heat stability further decreased. Essentially the same results were obtained also with citric acid (Fig. 4-2b) which, like EDTA, can be used to chelate calcium in these systems. Similar experiments with sweet wheys showed even lower heat stabilities when excess EDTA or citric acid was used (Fig. 4-3); this may indicate that in the very acidic environment with a relatively high amount of Ca^{2+} present as in the acid wheys, the chelation may not be complete and some of the calcium may still be available for stabilization of the protein against heat induced precipitation. The less variable content of phosphorus which is present in the acidic range mainly in the form of soluble phosphate (14) does not appear to have a direct effect on heat stability but presumably could be involved through partial effect on ionic strength of whey. With increasing ionic strength the solubility of whey protein is enhanced due to the formation of an ionic sheath around protein molecules. On the other hand, much higher increase of ionic strength leads to reverse effect - the decrease in the solubilization through "salting out" effect (13). In our experiments, the decrease in the heat stability of the tested wheys was indeed observed after increasing ionic strength by addition of either $CaCl_2$ or NaCl (data not reported).

When calcium was replaced with Na by passing the sweet or acid whey through an ion exchange resin, the heat stability was further lowered (Fig. 4-4) and both sweet and acid wheys behaved essentially alike. However, even the complete exchange of Ca by Na did not affect the high heat stability of these whey systems at pH 3.2 or below. To minimize the effect of different ionic strength, we used the Na-Ca exchange rather than total demineralization in these experiments.

While the overall differences in the heat resistance among the various systems examined appear relatively minor, they may be important for processors of whey drinks and similar products in which heat induced turbidity would be undesirable. An increase of 0.2 or even 0.1 pH unit in the critical pH range means relatively significant difference in sensory impact of these acidic beverages. As the results indicate the use of sweet wheys in whey beverages might be expected to present a somewhat greater potential for heat-induced protein coagulation than the use of acid wheys.

To check whether industrial cheese making procedures might result in substantial alterations of the heat stability patterns described above, wheys from one cheddar cheese manufacturer and two cottage cheese plants were collected twice at random order and included in our tests. The summary data are shown in Fig. 4-5. While the acid wheys show the expected higher heat stability when compared to the cheddar cheese whey, it is interesting to note the difference between the two acid wheys. One of the two cottage cheese wheys came from a plant using a high heat milk treatment for increased recovery of the whey protein in the cheese. As shown in Table 4-1, this proprietary treatment resulted in substantially lower protein content of the final whey and, correspondingly, in higher heat stability. It is somewhat surprising that despite the presumed removal of the heat coagulable whey protein in the cottage cheese, the whey still showed definite signs of heat-induced coagulation at pH 3.9-4.0 while little change in turbidity upon heating was noted at pH below 3.8. The second cottage cheese whey, obtained from a plant using a standard cottage cheese making procedure, behaved identically to the acid wheys tested earlier.

4.4. Conclusions

The measurements of turbidity change upon heating was used as a rapid and reproducible method for comparison of stability of various whey systems against heat-induced coagulation. Acid wheys showed generally higher heat stability than sweet wheys. This appeared to be related to their higher calcium content. The differences in the critical pH at which visible flocculation is expected to occur under the test conditions are small (3.9-3.8 for acid wheys vs 3.7-3.6 for sweet wheys) but reproducible with various laboratory-prepared or industrial products. At or below pH 3.5 all natural whey systems appear totally resistant to heat-induced turbidity changes. Differences in this range occured only after calcium was removed by ion-exchange or made unavailable by chelation. Since citric acid appeared to be as effective in chelating the Ca as EDTA, its use in industrial whey beverages processed by high heat treatment should be carefully monitored to avoid undesirable turbidity and coagulation.

4.5. Tables and Figures

Table 4-1: The composition and heat stability (critical pH range) of different types of industrial and laboratory prepared wheys investigated (average of at least two replicates).

Type of whey	pH initial	Total solids (%)	Protein (%)	Ca (ppm)	P (ppm)	pH range (20 min @ 92^{o}C)
Acid whey	4.61	7.20	0.75	1185	790	3.8-3.9
Sweet whey	6.60	6.54	0.90	325	400	3.6-3.7
Cheddar whey	6.05	6.56	0.84	455	490	3.6-3.7
Cottage cheese whey I	4.60	6.80	0.80	1320	815	3.8-3.9
Cottage cheese whey II	4.58	5.96	0.50	1200	810	3.9-4.0

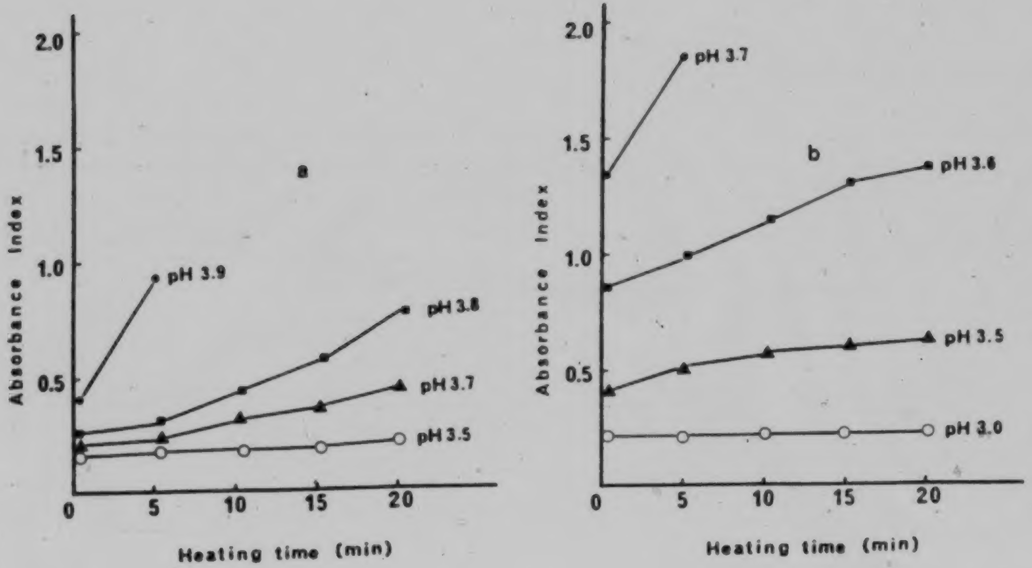

Figure 4-1. Changes in turbidity (absorbance index) of acid (a) and sweet (b) wheys upon heating at 92°C at various pH conditions.

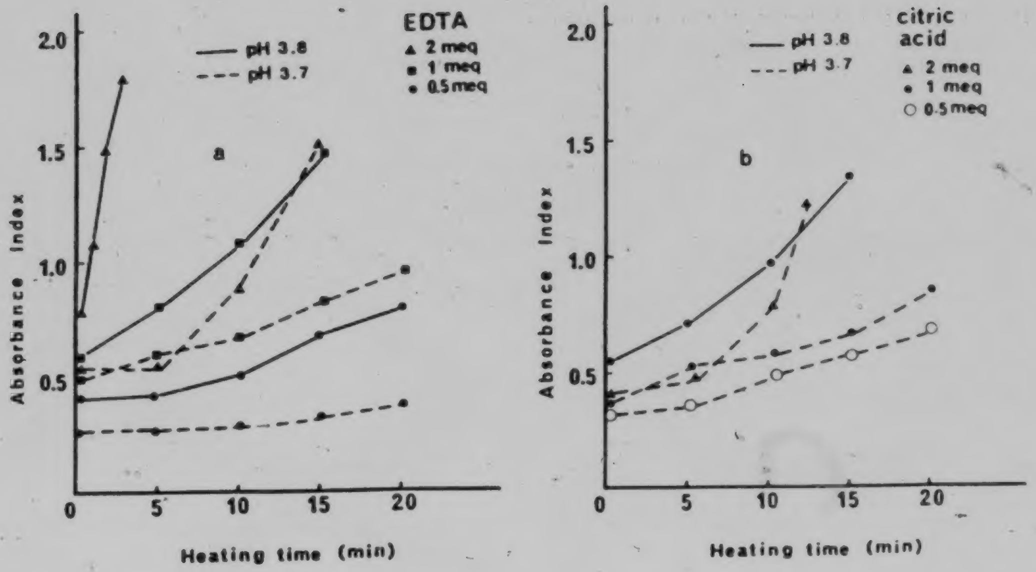

Figure 4-2. Turbidity (absorbance index) changes in acid wheys heated at 92°C with various amounts of EDTA (a) or citric acid (b).

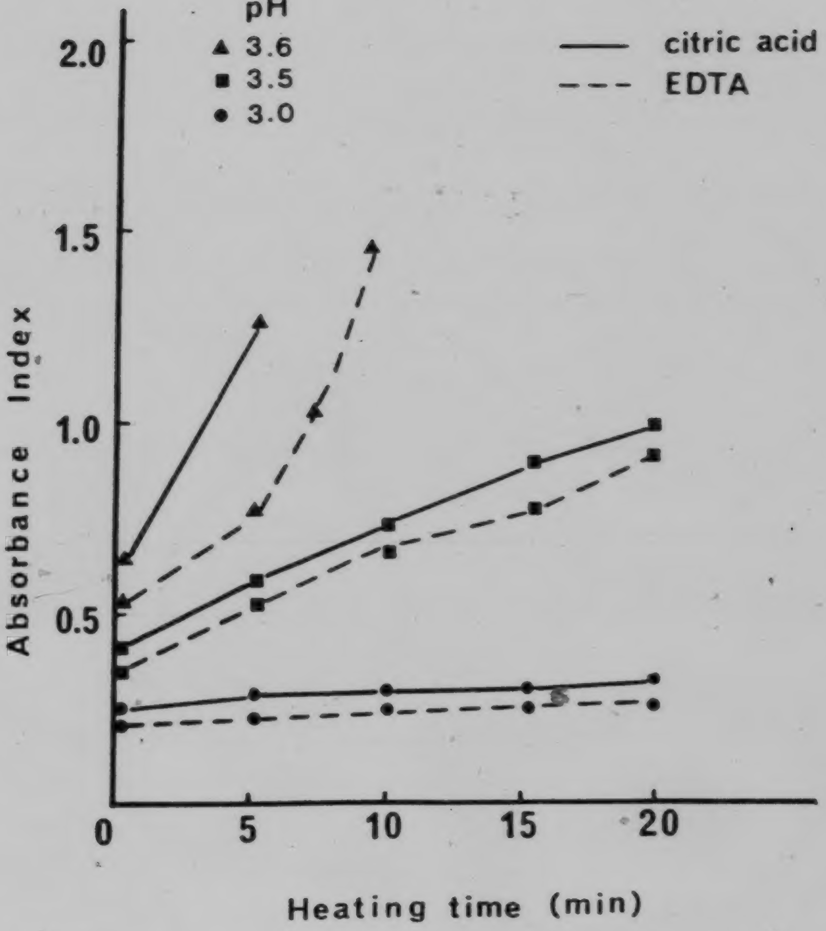

Figure 4-3. Heat-induced turbidity changes in sweet whey with 2:1 excess EDTA or citric acid based on Ca^{2+} equivalents.

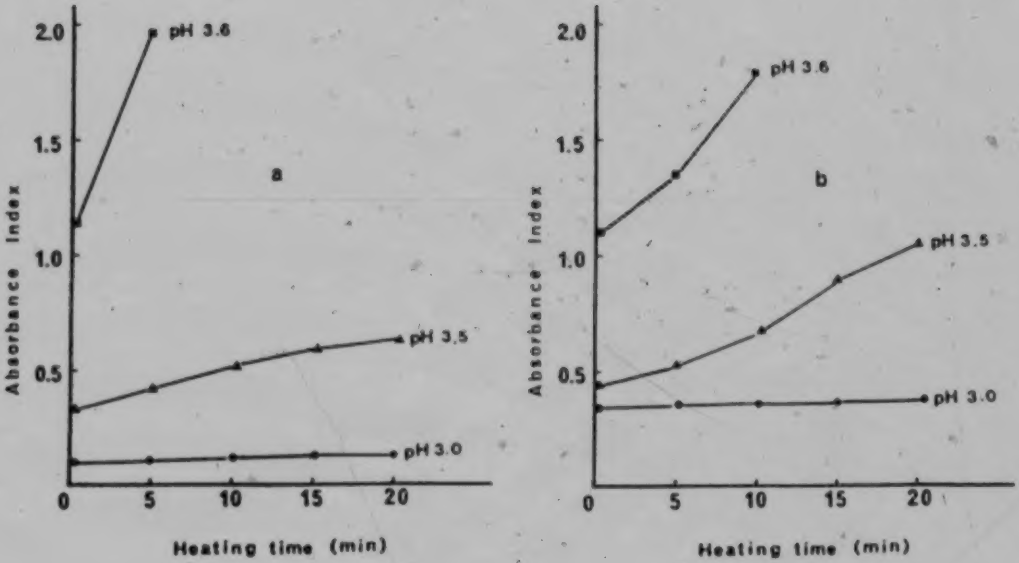

Figure 4-4. Effect of Ca^{2+} replacement by Na on heat-induced turbidity changes in acid (a) and sweet (b) wheys.

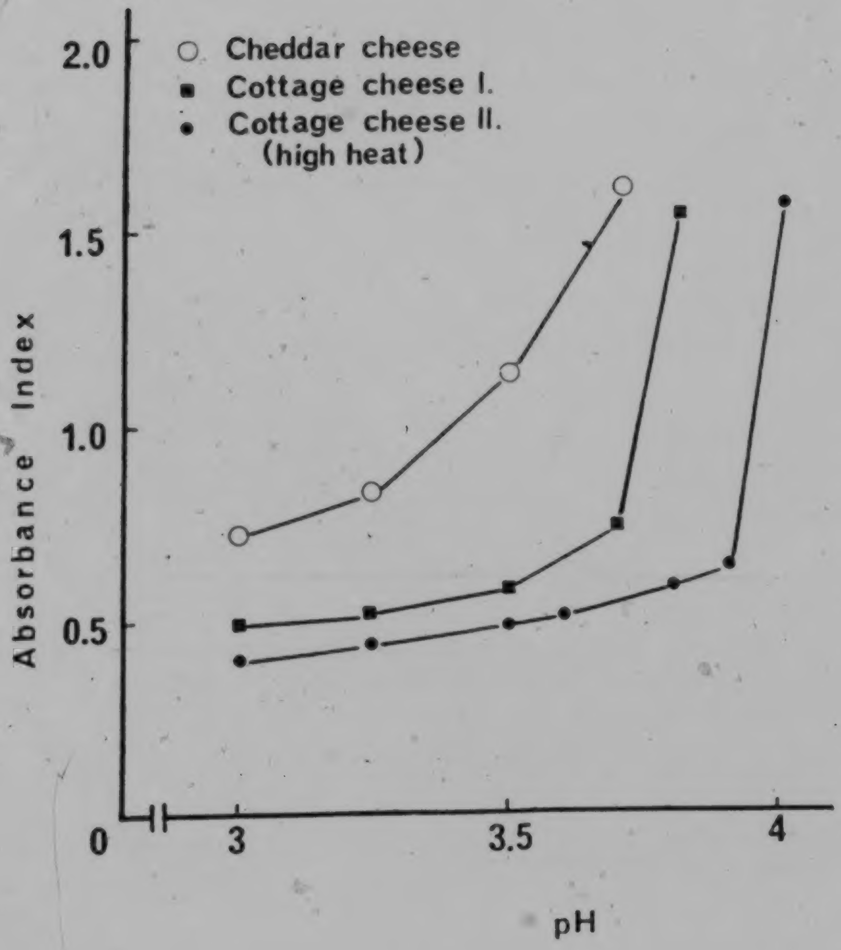

Figure 4-5. Changes in turbidity of industrial wheys heated for 10 min at 92°C and varying pH conditions.

4.6. References

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5. HEAT STABILITY OF ISOLATED WHEY PROTEIN FRACTIONS IN HIGHLY ACIDIC CONDITIONS[1]

5.1. Introduction

Heat sensitivity of whey proteins results in the formation of a precipitate upon heating of rennet (pH 6.5) or acid (pH 4.5) wheys above 70°C. The precipitate formation can be minimized by pH adjustment below the critical pH range of 3.7-3.9 (2, 5), depending on the calcium content (9). Although the denaturation temperatures of the individual whey proteins were determined in whey model systems (2,8), much less information is available on the heat resistance of the main whey protein fractions below the critical pH range. A recent literature review by Hill (3) indicates that while β-lactoglobulin (β-lg) appears to be resistant to heat coagulation after a conformational change from a dimer to a monomer at approximately pH 3.5, the α-lactalbumin (α-la) can be acid-denaturated at about the same pH. Heat denaturation of serum albumin (SA) is affected by the fatty acid contents (2). The effect of calcium on these changes in the acidic conditions of complex whey systems is not well understood.

The aim of this work was to study the behaviour of the individual whey proteins upon heating in ultrafiltration permeates of cottage cheese whey. Our objectives were (a) to determine the coagulation response of the individual protein fractions (β-lg, α-la, and SA) heated in highly acidic conditions separately and in combination simulating the composition found in whey; (b) to determine the effect of calcium removal from the permeate by ion exchange on the response of the individual proteins; and (c) to illustrate the protein coagulation behaviour by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

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[1] A version of this chapter has been published. G. Patocka, M. Drathen and P. Jelen 1987. Milchwissenschaft 42(11): 700 - 705

5.2. Materials and Methods

Experimental materials. Cottage cheese whey was obtained from a local dairy factory. To prepare the ultrafiltration (UF) permeate, the whey was processed in an Amicon UF module 8400 (Amicon Corp., Danvers, MA) using PM-10 flat sheet membranes (MW cut-off 10,000 D). Some of the permeate was passed through an ion exchange column filled with Bio-Rex 70 resin (Bio-Rad Laboratories, Richmond, CA) to remove calcium by replacement with sodium. The pH of the ion-exchange treated whey was readjusted after the treatment by lactic acid. Several batches of whey showing minimal compositional variability were used throughout this project.

To prepare the samples for the heating experiments, isolated whey proteins (Sigma Chemical Co., St. Louis, Mo) were dissolved in the whey permeates either individually or in combinations resembling typical concentrations found in cheese whey (0.4% β-lg, 0.2% α-la, and 0.1% SA) or in other concentrations as specified. Before heating, the pH of the solutions were adjusted to the desired values by 85% (v/v) lactic acid, using an Orion model

601A pH meter (Orion Research Inc., Cambridge, Mass.). The isolated whey protein preparations were used without further purification. Although some of the isolates contained traces of other whey proteins as seen on the electrophoretograms, this did not invalidate the main experimental observations.

Experimental procedures. Heating of all samples was carried out by placing 10 ml aliquots in covered test tubes into a boiling water bath. The maximum temperature achievable in the solutions (92°C) was reached in 180 ± 15 sec; the come-up time was subtracted from the data obtained. At the desired time intervals, test tubes were withdrawn, immediately cooled in an ice bath, and after temperization to 20°C, turbidimetric measurements of absorbance index (AI) were carried out using an EQA-1 (Gaulin Corp., Everett, Mass.) 900 nm wavelength spectrophotometer. The details of the methodology were described earlier (5).

Slight variations in turbidity data of unheated samples were observed as a result of the varying protein concentrations used and the effect of the ion exchange treatment; thus, turbidities of the unheated samples were subtracted from the AI values determined after heating. All turbidimetry experiments were repeated at least twice. Minor inconsistencies in the AI values obtained in the replicate experiments did not affect the general trend observed.

Chemical analyses and SDS-PAGE. Calcium and phosphorus contents of the permeates used were determined by atomic absorption with the AtomScan 2000 Spectrophotometer (Jarrel Ash Div., Fisher Scientific Co., Waltham, Mass.), while protein (N x 6.38) and total solids were obtained by routine analytical methods (Patocka et al., 1986). Selected heated and unheated samples were subjected to SDS-PAGE slab electrophoresis using the Mini-Protean II Slab Cell (Bio-Rad Laboratories, Richmond, CA) and the standard procedures described earlier (6,7). The 1.5 mm thick gels were run at constant voltage of 200 V for 40 minutes, stained and destained for 12 hr and preserved by drying with the use of cellophane membranes.

5.3. Results and Discussion

Analytical characteristics of the UF cottage cheese whey permeates used are shown in Table 5-1. Besides the effective calcium removal, the ion exchange process slightly decreased the nitrogenous matter content while the effect on phosphorus was negligible.

The heat stability of the individual whey proteins was tested in regular as well as in virtually calcium-free permeates. The response of the β-lg (Fig. 5-1) was similar to that observed earlier (9) for whole wheys. At 0.4% concentrations, heating at 92°C in regular permeates produced gradually increasing turbidity at pH 3.8 or higher while at pH 3.7 or below, the turbidity change was minimal. After calcium removal from the permeate, rapid turbidity increase was observed at pH 3.7, while at pH 3.8, the solution flocculated in less than 5 min. The critical pH for the rapid turbidity development in the β-lg solution was also protein concentration dependent; at 0.2% β-lg, the critical pH for rapid turbidity development increased to 3.9, while at 0.1% β-lg, the onset of turbidity was not observed until pH 4.1.

The SDS-PAGE results (Fig. 5-2) confirm the AI determinations indicating that in regular, calcium-containing permeate, the 0.4% β-lg solution was fully resistant to coagulation at pH 3.7, while in the absence of calcium, heating for 15 min at 92^{o}C substantially removed the β-lg from the PAGE pattern. In the pH 4.0 and 4.2 electrophoretograms the gradual disapperance of the β-lg bands is consistent with the overall trends indicated by the AI measurements.

The behaviour of α-la and SA at their respective concentrations was quite different from that of the 0.4% β-lg solutions (Fig. 5-3). In the Ca-containing permeate, the isolated α-la appeared completely resistant to heat coagulation; no observable turbidity developed upon heating at any pH up to the normal unadjusted permeate (pH 4.5). Likewise, the electrophoretic pattern (Fig. 5-4A) failed to indicate any effect of heating on the removal of the protein. In the Ca-free permeate, increased turbidity was observed at the onset of the heating but the turbidity did not change substantially as the heating progressed, and the PAGE pattern (Fig. 5-4B) did not show any substantial effect of heating. While the apparent resistance of α-la to heat coagulation at pH 3.7 or higher confirms our previous DSC data (1), the lack of disappearance of the α-la bands in the Fig. 5-4B is somewhat surprising in view of the irreversibility of the denaturation reaction in the absence of free calcium (1).

The SA at 0.1% concentration behaved similarly to the α-la, producing a slight heat induced turbidity only at pH 4.2 with little effect of heating time (Fig.

5-3). The SDS-PAGE patterns of the isolated protein again confirmed stability against heat precipitation in the whole pH range investigated (Fig. 5-5A). In calcium free permeate the isolated SA was heat stable at pH 3.7, but was removed from the solution at pH 4.2 after heating (Fig. 5-5B).

Increasing the protein concentration of either α-la or SA to 0.4% had relatively little effect on the observed turbidity development in the calcium containing permeates. With α-la no effect was observed up to pH 4.5, while the 0.4% solution of SA showed strong coagulation tendency at pH 4.3 or higher.

When the three isolated proteins were dissolved and heated together in a regular permeate in the combination resembling the typical protein concentration in cheese whey (0.4% β-lg, 0.2% α-la, 0.1% SA), rapid increase in turbidity was observed at the critical pH range of 3.8-3.9, exactly as reported by Patocka et al. (9) for cottage cheese whey. The PAGE patterns (Fig. 5-6) again confirmed the dependence of the heat sensitivity of the β-lg on pH. However, in contrast to the PAGE patterns of the isolated proteins, Fig. 5-6 indicates some possible precipitation of α-la and SA with the β-lg at pH 4.0 and especially pH 4.2. It may be that the aggregation of the β-lg could include non-specific reactions with SA and α-la which, in the absence of β-lg, appeared resistant to heat induced turbidity development in isolated Ca-containing permeate systems (Fig. 5-3). Similar observations were also noticed with regular cottage cheese whey at pH 4.0 and higher (Fig. 5-7A), while in the ion-exchange treated cottage cheese whey (Fig. 5-7B) the same β-lg band disappearance from patterns can be observed already at pH 3.8 in accordance with the decreased β-lg heat stability (Figs. 5-1 and 5-2).

5.4. Conclusions

As expected, the β-lg showed the greatest variation in the heat-induced precipitation behaviour with changes in pH. At pH 3.7, the effect of heating at 92^{o}C for 15 min appeared minimal with all three proteins; at pH 4.0 and 4.2, the β-lg and SA were almost completely removed from whey or model protein mixtures while α-la concentration decreased only slightly. The apparent heat resistance of α-la in the calcium-containing solutions is obviously related to its ability to renature despite its low heat denaturation temperature (1).

The heat coagulability of β-lg in the critical pH range appears to be strongly influenced by calcium. After removing the Ca from the permeate, the gradually increasing turbidity appeared at a pH as low as 3.6. The stabilizing effect of calcium on β-lg has been indicated by Hillier et al. (4). Qualitative effects of calcium removal on the individual isolated proteins are shown in Figures 5-2, 5-4 and 5-5; however, whether the same effects can be used to explain the observed anomalies in the combination systems (Figs. 5-6 and 5-7) is not known. It appears that when all the main whey proteins are present, the co-precipitation of the α-la and especially SA with the heat-sensitive and abundant β-lg occurs. In the acid whey systems this may result from the increased availability of Ca-binding active sites of the various protein molecules. The main determinant of heat stability of whey systems heated at very acidic conditions appears to be the behaviour of β-lg.

5.5. Tables and Figures

Table 5-1. Total solids, total nitrogen, calcium and phosphorus content of regular and ion-exchange treated Cottage cheese whey UF permeate.

Component	UF Permeate			
	Untreated		After ion-exchange	
	average	range	average	range
Total solids	5.19	(4.92-5.39)	4.83	(4.54-5.12)
Total nitrogen	0.04	(0.041-0.047)	0.03	(0.025-0.031)
Calcium	0.10	(0.09-012)	0.003	(0.002-0.005)
Phosphorus	0.07	(0.07-0.08)	0.06	(0.06-0.07)

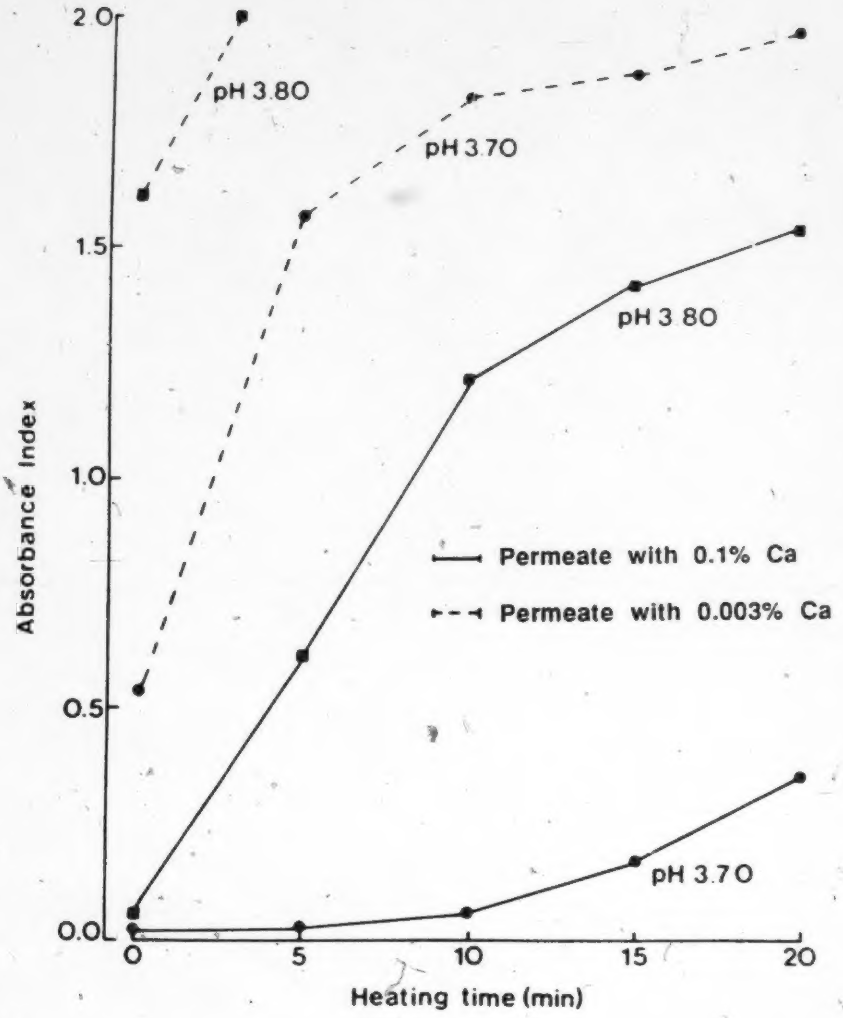

Figure 5-1. Heat stability of 0.4% β-lg solution in cottage cheese whey UF permeate with the normal and reduced Ca content.

Figure 5-2. SDS-PAGE electrophoretograms of 0.4% β-lg solution in cottage cheese whey UF permeate with normal (A) and reduced (B) Ca content:

a - pH 3.7 heated; b - pH 3.7 unheated; c - pH 4.0 heated; d - pH 4.0 unheated; e - pH 4.2 heated; f - pH 4.2 unheated.

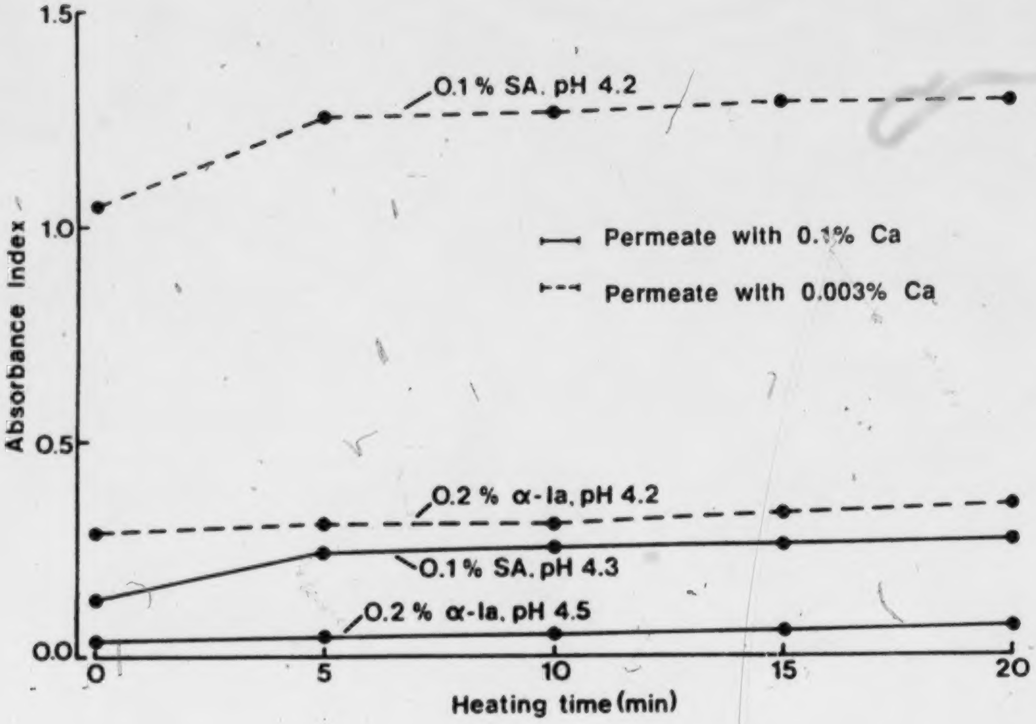

Figure 5-3. Heat stability of 0.2% α-la and 0.1% SA solutions in cottage cheese whey UF permeate with normal and reduced Ca content.

Figure 5-4. SDS-PAGE electrophoretograms of 0.2% α-la solution in cottage cheese whey UF permeate with normal (A) and reduced (B) Ca content:

a - pH 3.7 heated; b - pH 3.7 unheated; c - pH 4.0 heated;

d - pH 4.0 unheated; e - pH 4.2 heated; f - pH 4.2 unheated

g - reference cottage cheese whey UF permeate, pH 4.5 unheated (A) and heated (B).

Figure 5-5. SDS-PAGE electrophoretograms of 0.1% SA solution in cottage cheese whey UF permeate with normal (A) and reduced (B) Ca content:

a - pH 3.7 heated; b - pH 3.7 unheated; c - pH 4.0 heated;
d - pH 4.0 unheated; e - pH 4.2 heated; f - pH 4.2 unheated.

Figure 5-6. SDS-PAGE electrophoretograms of 0.1% SA, 0.2% α-la and 0.4% β-lg mixture in cottage cheese whey UF permeate: a - pH 3.7 heated; b - pH 3.7 unheated; c - pH 4.0 heated; d - pH 4.0 unheated; e - pH 4.2 heated; f - pH 4.2 unheated.

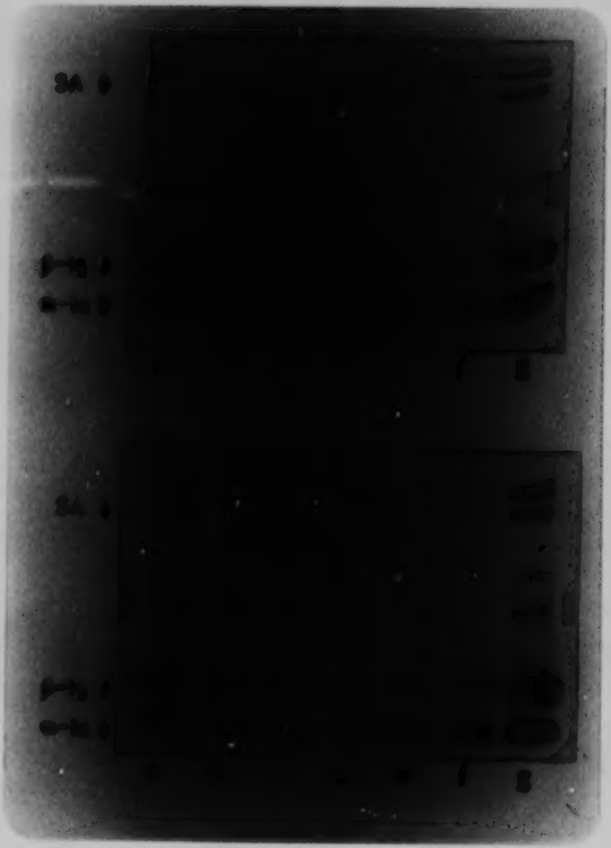

Figure 5-7. SDS-PAGE electrophoretograms of cottage cheese whey before (A) and after (B) Ca ion exchange treatment: a - pH 3.7 unheated; b - pH 3.7 heated; c - pH 3.8 heated; d - pH 4.0 heated; e - pH 4.2 heated; f - reference whey (pH 4.5) heated; g - reference whey (pH 4.5) unheated.

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6. EFFECT OF pH AND CALCIUM ON THERMAL BEHAVIOUR OF ISOLATED WHEY PROTEINS[1]

6.1. Introduction

The solubility of whey proteins is an important functional characteristic affecting their suitability for incorporation into various food systems. However, as heating is a major technological operation in dairy processing, heat induced insolubilization of the heat sensitive whey proteins could result in substantial changes of functionality. Numerous studies have been concerned with the thermal behaviour of individual whey proteins with a special attention being devoted to the study of relationships between the heat coagulation and environmental factors such as pH, presence of minerals and protein concentration.

The excellent heat stability of whey proteins in highly acidic conditions was illustrated in our earlier work (Patocka et al., 1986). Mild heat treatment beyond the isoelectric pH zone will not alter protein solubility; however, more severe heating results in protein precipitation (de Wit and Klarenbeek, 1984). The calcium involvement in heat coagulation of whey proteins was demonstrated by de Rham and Chanton (1984) and studied in detail by Donovan and Mulvihill (1987). As indicated in Mulvihill and Donovan's (1987) comprehensive review on whey protein thermal denaturation, the heat response of individual whey proteins varies. Differential scanning calorimetry measurements showed that the denaturation temperatures of the dominant whey protein fractions β-lactoglobulin (β-lg) and α-lactalbumin (α-la) are different (Bernal and Jelen, 1985). The α-la is heat stable at its isoelectric pH (4.2-4.5); however, in the presence of β-lg, both proteins co-precipitate upon heating (Patocka et al., 1987). The heat coagulation of β-lg is pH dependent; at neutral pH the precipitation is controlled by presence of divalent cations. In contrast, α-la seems to be insensitive to Ca induced coagulation in the 2-12 pH range. However, it was pointed out that the behaviour of isolated proteins cannot be simply extrapolated to "real" whey systems (Townend and Gyuricsek, 1974). Recently Hill (1988) proposed a mechanism of thermal response of major protein fractions in cheese whey. In his interpretation the β-lg thermocoagulability is pH dependent and the influence of Ca becomes dominant above the protein isoelectric pH (5.2-5.4). The heat induced β-lg-α-la interaction and co-precipitation plays a major role in α-la insolubilization.

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[1] A version of this chapter has been submitted for publication. G. Patocka and P. Jelen 1990. Submitted to Journal of Food Science.

Our previous paper (Patocka et al., 1987) dealt with coagulation response of isolated whey proteins in whey systems at pH below 4.5. The objectives of the present study were to characterize and compare the thermocoagulation response of isolated α-la and β-lg heated separately and in a model mixture within the 4.5-7.0 pH range in regular or Ca depleted cottage cheese whey ultrafiltration (UF) permeates.

6.2. Materials and Methods

Industrial cottage cheese whey (Palm Dairies, Edmonton) was ultrafiltered on an Amicon UF cell (Amicon Corp., Danvers, MA) using a PM-10 membrane with 10,000 D molecular weight cut-off. The absence of whey proteins from the permeate was checked electrophoretically by SDS-PAGE (Patocka et al., 1987). A portion of the permeate was decalcified by passing through an ion exchange column packed with weekly acidic cationic exchanger BioRex 70 (BioRad Laboratories, Richmond, CA). The experimental pH of the permeate was adjusted by 2N NaOH or 2N HCl. The Ca phosphate which precipitated above pH 6 was separated by centrifugation on a laboratory centrifuge IEC HN-S II (Damon/IEC Div., Needham, Mass.) at 5,300 x g for 10 min and the supernatants were filtered through a filter paper. Where necessary, the Ca content was standardized by 1M $CaCl_2$ (BDH Inc., Edmonton, Alberta).

The isolated proteins (Sigma Chemical Comp., St. Louis, Mo) were dissolved in permeates at concentrations typically found in cheese whey (0.4% β-lg, 0.2% α-la). All heating experiments were carried out in 10 ml quantities using test tubes covered with Al foil and held in a thermostatically controlled water bath at $93\pm1^{o}C$. Heating time was taken from the time the samples reached the desired temperature as indicated by a thermocouple inserted into a control sample. After heating for defined time intervals samples were cooled in an ice bath, equilibrated at $20\pm1^{o}C$ and assayed for absorbance by measurements on a B-L Spectronic 21 spectrophotometer (Fisher Scientific, Pittsburgh, PA) at wavelength of 900 nm. Preliminary experiments showed complete compatibility with absorbance values measured on the EQA-Digital Analyzer, which was used in our earlier studies (Patocka et al., 1986; 1987). Heating experiments were repeated three times and all absorbance values were obtained as means of three replicates.

The Ca content in the permeates was determined by atomic absorption according to Brule et. al (1974) using a Perkin-Elmer atomic absorption spectrophotometer (Perkin-Elmer Corp., Norfolk, CT). Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was used to identify individual major proteins in unheated and heated solutions and, where applicable, in supernatants and corresponding precipitates. The precipitates were separated from the solutions by filtration and washed thoroughly with distilled water, until no protein was detectable in the filtrate. About 1 mg of each individual precipitate was solubilized in 10 ml of a reducing buffer (aqueous solution of 10% SDS, 0.05M Tris pH 6.8, 5% 2-mercaptoethanol, 5% glycerol) in a boiling water bath for 5 min. A standard SDS-PAGE procedure used with all samples was as described earlier (Patocka et al., 1987).

6.3. Results and Discussion

α-lactalbumin

The heat stability of α-la was tested in a 0.2% concentration in regular or decalcified cottage cheese whey UF permeate at pH ranging from 4.5 to 7.0.

As indicated by the absorbance measurements the changes in thermal behaviour became apparent at pH 5.5 (Fig. 6-1A). Further increase of pH to 6.0 resulted in the formation of a precipitate after approximately 10 min heating. However, in parallel experiments using UF permeate with Ca content standardized by $CaCl_2$ addition to the level determined at pH 4.5 (Table 6-1), all heated solutions above pH 5.5 flocculated rapidly after 1-3 min heating.

The precipitation response of α-la was changed profoundly after complete Ca removal from the permeate. No heat imparted coagulation was observed in the whole experimental pH range of 4.5-7.0 (Fig. 6-1B). The noticeably higher absorbance at pH 4.5 in comparison to that observed in regular heated permeate indicated an alteration of protein solubility (Fig. 6-2). Nevertheless, this partial insolubilization reflecting Ca unavailability in the solution (Bernal and Jelen, 1984) did not result in protein sedimentation after cooling. As pH progressed to the more neutral region, the intensity of the heat-induced turbidity fainted. Above pH 6.5 the differences in absorbance recorded at the beginning and at the end of the heating period were insignificant.

This variation of α-la heat stability, which contradicts a mechanism of precipitation proposed by Hill (1988), indicated a direct Ca involvement in protein insolubilization. However, in contrast to β-lg, the Ca-triggered mechanism of observed aggregation cannot be simply explained by electrostatic effects, because of the protein solubility in the isoelectric pH zone.

It appears that the high negative charge (Kronman and Andreotti, 1964) together with increased extent of Ca-protein binding (Patocka and Jelen, 1990) caused gross aggregation of the heat denatured protein aided by Ca bridging (Donovan and Mulvihill, 1987) resulting in the protein insolubilization.

β-lactoglobulin

Between pH 4.5-6.5 the solutions of β-lg (0.4%) in regular permeate flocculated just before reaching 93°C. The coagulation produced very distinct, sharply defined aggregates floating in clear liquid. However, no precipitation was observed during 30 min heating at pH 7.0, and the differences in recorded absorbance readings were minimal (Fig. 6-3A). The enhanced heat stability at this pH appeared to reflect the low amount of Ca present in the solutions (Table 6-1). When the Ca level in the pH 7.0 sample was equalized to that found at pH 4.5, the solution became heat unstable and the β-lg precipitated regardless of pH.

After Ca removal from the permeate no signs of heat induced coagulation were evident at pH 6.5 and 7.0. The excellent heat stability of β-lg at this pH zone at the reduced Ca concentration of our system is comparable with observations made earlier (de Wit, 1981; Jelen and Patocka, 1989). Below pH 6.0, however, the heat stability did not improve even after complete decalcification (Fig. 6-3B).

α-lactalbumin/ β-lactoglobulin

When both isolated proteins, α-la and β-lg, were heated together in either regular or decalcified permeates in concentrations of 0.2 % and 0.4%, respectively, the observed precipitation characteristics were identical to that of β-lg. As expected, SDS-PAGE of supernatants from the heated solutions showed disappearance of the β-lg band from the patterns (Fig. 6-4a). However, fainting of bands representing α-la and the presence of those bands in the patterns of precipitates (Fig. 6-4b) indicated a partial insolubilization of this protein as well, although when heated alone, no loss of solubility was observed between pH 4.5 - 5.0.

A similar trend was seen after heating the protein mixture in a decalcified permeate (Fig. 6-5a). Above pH 6.0, the protein solubility remained unaffected and corresponding electrophoretic patterns (slots 5 and 6) did not reveal any change compared to unheated samples (slot 7). With pH progressing to acid regions (slots 1-4), disappearance of β-lg from the patterns became evident together with slightly diminishing α-la bands which appeared again in the protein profile of the precipitates (Fig. 6-5b).

The slow electrophoretic mobilities of the solubilized whey protein aggregates were enhanced by up to 5% addition of a reducing agent (2-mercaptoethanol) to the buffer solution. After this treatment, the individual protein zones in the electrophoretic patterns became identical to PAGE protein profiles of unheated solutions. The electrophoretograms of separated and dissolved precipitates demonstrated clearly the dominance of the β-lg in the precipitate; nevertheless, the apparent presence of α-la bands in the PAGE patterns indicates some α-la co-precipitation. This is in a sharp contrast to the behaviour of α-la heated alone when no sign of precipitation was observed after heating at pH between 4.5-7.0 in decalcified or 4.5-5.0 in regular permeate. It appears that the presence of β-lg in the solution affects the stability of α-la, which results in partial α-la precipitation. However, whether this precipitation happened via interaction and/or formation of co-aggregates between both proteins could not be ascertained because of the resolubilization of the precipitates before the SDS-PAGE analysis, especially due to the breakdown of disulphide bonding of the aggregates (Sawyer, 1968) by the chemical dissociating agent 2-mercaptoethanol.

6.4. Conclusions

Our observations demonstrated that the interrelationship between Ca and pH is a major factor affecting aggregation of whey proteins upon heating. The isolated β-lg showed high heat sensitivity resulting in loss of solubility between pH 4.5 and 6.0 regardless of Ca availability. Under similar conditions α-la appeared quite heat resistant towards heat induced insolubilization below pH 5.0. The solubility of the α-la above pH 5.5 and of the β-lg above pH 6.5 improved at reduced Ca concentrations and the proteins appeared to be heat stable. When heated together the precipitation characteristic of β-lg did not change; however, α-la appeared to follow a different precipitation mechanism influenced by the β-lg presence, with both proteins co-precipitating.

6.5 Tables and Figures

Table 6-1. Total Ca content in pH adjusted UF permeates and initial turbidity of solubilized proteins.

	pH					
	4.5	5.0	5.5	6.0	6.5	7.0
	Ca (ppm)[c]					
UF permeate[a]	1050 ± 23			947 ± 17	799 ± 13	259±8
	turbidity (A_{900})[c]					
0.4% β-lg[b]	< 0.1					
0.2% α-la	0.15 ± 0.02	0.10 ± 0.02	< 0.1			
0.2% α-la decalcif.	0.28 ± 0.03	0.25 ± 0.03	< 0.1			

[a] Ca content of decalcified UF permeate was < 0.02 ppm

[b] measured absorbances in regular or Ca reduced permeates were not significantly different

[c] means of nine replicates ± SD

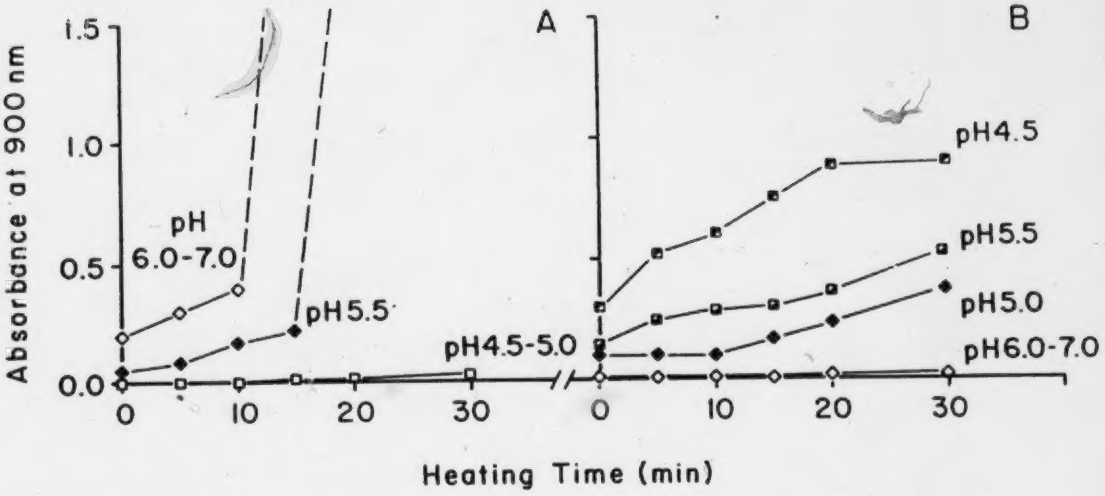

Figure 6-1. Heat stability of 0.2% α-lactalbumin in cottage cheese whey UF permeate with normal (A) and reduced (B) Ca content.

----- precipitation

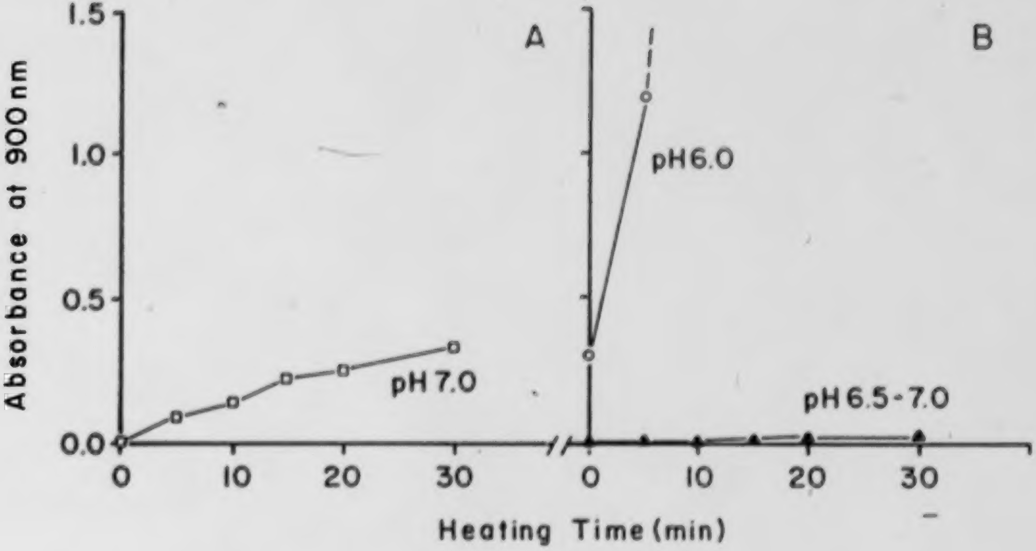

Figure 6-3. The heat stability of 0.4% β-lactoglobulin in cottage cheese whey UF permeate with regular (A) and reduced (B) Ca content.

----- precipitation

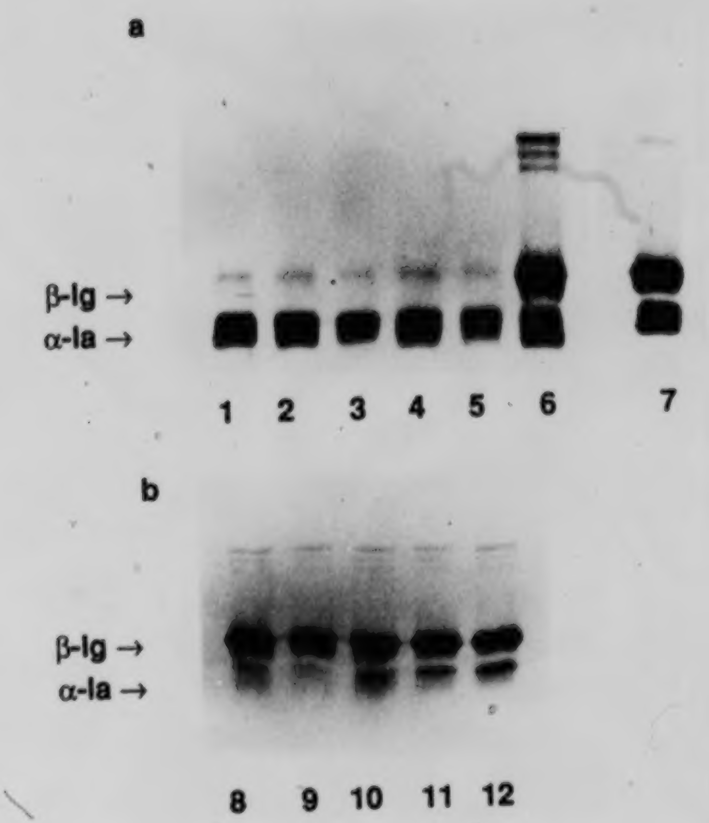

Figure 6-4. SDS-PAGE electrophoretograms :
(a) 0.2% α-la and 0.4% β-lg solution in regular cottage cheese whey UF permeate heated for 30 min at 93±1°C;
slot 1 - pH 4.5; slot 2 - pH 5.0; slot 3 - pH 5.5; slot 4 - pH 6.0; slot 5 - pH 6.5; slot 6 - pH 7.0; slot 7 - unheated solutions at pH 4.5 (all other patterns of unheated samples up to pH 7 were identical);
(b) separated solubilized precipitates;
slot 8 - pH 4.5; slot 9 - pH 5.0; slot 10 - pH 5.5; slot 11 - pH 6.0; slot 12 - pH 6.5.

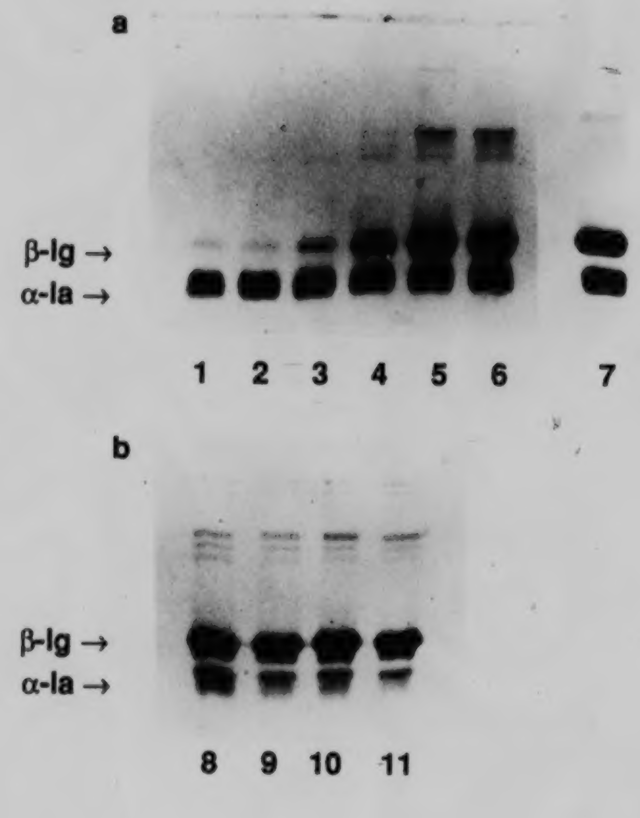

Figure 6-5. SDS-PAGE electrophoretograms :
(a) 0.2% α-la and 0.4% β-lg solution in decalcified cottage cheese whey UF permeate heated for 30 min at 93±1°C; slot 1 - pH 4.5; slot 2 - pH 5.0; slot 3 - pH 5.5; slot 4 - pH 6.0; slot 5 - pH 6.5; slot 6 - pH 7.0; slot 7 - unheated solutions at pH 4.5 (all other patterns of unheated samples up to pH 7 were identical);
(b) separated solubilized precipitates;
slot 8 - pH 4.5; slot 9 - pH 5.0; slot 10 - pH 5.5; slot 11 - pH 6.0.

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7. BINDING OF CALCIUM TO ISOLATED WHEY PROTEINS[1]

7.1. Introduction

The major whey protein fractions, α-lactalbumin (α-la) and β-lactoglobulin (β-lg), have been the subject of a considerable interest in both fundamental and applied research. Since it was found that bovine α-la is capable of binding calcium (Hiraoka et al., 1980), there has been thorough investigation of the physico-chemical aspects of these calcium-protein interactions (Kronman et al.,1981; Permyakov et al., 1982; Murakami et al., 1982). The protein in its native form contains one or two Ca ions per mole, but the location of the binding sites and the extent of binding remain ill-defined.

Much less information is available on Ca binding to β-lg. Carr (1953) evaluated the Ca binding capacity of β-lg at pH 7.4. The amount of Ca bound to the β-lg was suggested to be equivalent to the protein charge (Zittle et al., 1957). A more recent investigation revealed that β-lg interactions with minerals were influenced by physico-chemical characteristics of a particular system (Baumy and Brule, 1988); in aqueous phase, the protein affinity for divalent cations became lower when pH decreased or when ionic strength increased.

Calcium-whey protein relationships are highly dependent on environmental factors (de Wit and Klarenbeek, 1984); this has direct relevance for the development of various whey processing techniques (Patocka and Jelen, 1987) and for functionality of the whey proteins (Morr, 1982). The calcium content in whey plays a major role in the stabilization of a whey protein system against heat induced precipitation (Patocka et al., 1986, 1987; de Rham and Chanton, 1984). Calcium modulates also the heat stability of milk with modified casein-whey protein content as indicated by recent investigations (Jelen and Patocka, 1989).

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[1] A version of this chapter has been submitted for publication. G. Patocka and P. Jelen 1990. Submitted to CIFST Journal.

The aim of this study was to contribute to the elucidation of interactions between calcium and the main whey proteins in a wide range of environmental conditions approaching closely the changing characteristics of "real" whey systems subjected to various whey processing techniques. The specific objectives were: (1) to evaluate the effects of pH and ionic strength on the Ca binding capability of α-la and especially β-lg in aqueous solutions, and (2) to determine Ca-binding responses of the two whey proteins in a whey UF permeate.

7.2. Materials and Methods

Materials. Isolated α-lactalbumin and β-lactoglobulin preparations were obtained from the Sigma Chemical Company (St.Louis, Mo). Various reagent grade chemicals used for the buffer preparations included acetic acid, sodium acetate, NaCl, NaOH and HCl (BDH Inc., Edmonton, Alberta), cacodylic acid (Sigma), Tris and glycine (BioRad Laboratories, Richmond, CA), while $CaCl_2$ (2 H_2O) used in the binding study was obtained from BDH. The ion-exchange resins BioRex 70 and AG 3-X4A were from BioRad; the gel filtration was performed with Sephadex G-100 (Pharmacia, Dorval, Quebec). Double distilled quality water was obtained by passing tap water through a Millipore purification system (Millipore Corp., Redford, MA).

Demineralization of isolated proteins. The calcium free α-lactalbumin was prepared by demineralization of an α-la preparation from Sigma (L-5385 type I; lot 124F-8086) following a method described by Kronman and Bratcher (1983). A solution of 3 g protein in 200 ml of 0.002 M Tris buffer was adjusted to pH 1.7 by 2N HCl and diafiltered on an Amicon 8400 UF cell with PM 10 membrane (M.W cut-off 10,000 D) using an Amicon UF/DF selector valve (Amicon Corp., Danvers, MA), first against the buffer to remove bound minerals and finally against distilled water. The diafiltration was followed by ultrafiltration (UF) to the final volume of 30 mL; the protein concentrate was then freeze dried.

The β-lg (5g) from Sigma (L-2506; lot 52F-8035) was dissolved in 300 mL of 0.002 Tris buffer, acidified to pH 2.5 with 1N HCl and treated as above.

Experimental protein solutions. The 0.2% α-la solutions were prepared by dissolving demineralized protein isolate (Sigma) in buffers of desired pH and ionic strength. The following buffers were used: HCl-glycine buffer (pH 3.5), acetate buffer prepared from acetic acid and sodium acetate (pH 4.0 - 5.5) and cacodylic buffer prepared from cacodylic acid and NaOH (pH 6-7). The mixing ratio of buffer components to provide the ionic strength of 0.04 was as described (Davidek, 1977). The experimental range of ionic strengths was chosen to approach the estimated milk ionic strength (Walstra and

Jenness, 1984) and to reflect changes in ion composition, pH and concentration, which are typical for processed whey. The required ionic strength was obtained by the addition of sodium chloride. The desired level of Ca concentration was adjusted with the $CaCl_2$ standard solution (1 mg Ca/ml). The β-lg stock was prepared in 0.4% concentration in the same way.

Experimental samples (30 ml aliquots) were equilibrated for 4 h at 20±0.5°C and subsequently ultrafiltered in an Amicon PM50 UF cell using PM-10 membranes (M.W. cut-off 10,000 D; diam. 43 mm) and driving pressure of 0.3 bar (nitrogen); at such a low pressure the flux decline effects of membrane compaction were minimal (Tarnawski and Jelen, 1986). The outflow was recycled for about 1 hr until Ca content in the permeate was constant, at which point samples from permeate and retentate side were collected and analyzed. The protein absence in the permeate was checked by SDS-PAGE at the end of individual experimental runs. At the beginning of all experiments the UF membrane was saturated with calcium using protein-free buffer until Ca level on retentate side was equal to that of permeate side to minimize errors resulting from calcium absorption by the membrane.

Protein separation from acid whey. Industrial cottage cheese whey (Palm Dairies, Edmonton) was clarified on a laboratory centrifuge (J2-21, Beckman Instruments, Palo Alto, CA) to remove casein fines (Patocka and Jelen, 1989) and ultrafiltered on an Amicon 8400 UF cell using PM-10 membranes to 15% total solids content. The UF retentate was partially desalted by dialfitration and freeze dried. The gel filtration procedure of de Wit and Klarenbeek (1984) was used for protein separation. The freeze-dried UF whey concentrate was dissolved in a phosphate buffer from Sigma (0.1M, pH 6.5) in 0.1g/ml concentrations and subsequently fractionated on a Sephadex G-100 packed column (5 ml sample, column 75x2.5 cm, flow 0.5 ml/min, 2 ml fractions). The elution profile was monitored by absorbance reading at 280 nm using the Beckman DU-8 spectrophotometer (Beckman Instruments, Palo Alto, CA). Individual protein peak fractions were evaluated by SDS-PAGE according to methodology described earlier (Patocka et al., 1987). Selected fractions containing separated α-la and β-lg were pooled and freeze dried. Lyophilizates were finally demineralized as above and freeze dried again.

Preparation of partially demineralized whey systems. The method described by de Rham and Chanton (1984) was adopted for preparation of selectively demineralized whey permeates. The treatment of whey UF permeate followed by mineral adjustment was necessary for the experiments to be conducted in a feasible way. The presence of large amounts of phosphates, citrates and lactates in the whey together with the high affinity of these whey components for calcium makes it very difficult to determine exactly the proportion of calcium tied to the proteins. Our use of acid whey permeate provided a model system simulating whey with simple control of desired calcium level. The acid whey UF permeate was passed through two ion-exchange columns in sequence. The first column was cationic, with BioRex 70 resin in Na cycle designed to replace calcium by sodium. The second column was anionic, filled with AG3-X4A resin in chloride form for removing mineral anions and organic acids. The ion-exchange treatment was followed by neutralization to pH 7 using KOH.

Preparation of 0.2% α-la and 0.4% β-lg solutions was in 100 mL aliquots using the laboratory-prepared acid whey protein isolates. The required Ca concentration was accomplished by the addition of $CaCl_2$ standard solution (1 mg Ca/ml). The pH of individual samples was adjusted by either 2N HCl or 2N NaOH. Samples were equilibrated and ultrafiltered under the same conditions as above.

Determination of bound calcium. According to Baumy and Brule (1988) the amount of protein-bound calcium was determined as a difference between Ca in UF retentate and Ca in UF permeate. In the conditions of low protein concentrations and the presence of an electrolyte, as used in our experiments, the Donnan binding effect is considered to be negligible (Hughes and Klotz, 1956).

The extent of calcium-protein binding was expressed as

$$b_e = Ca_b/P \qquad (1)$$

where Ca_b is the concentration of protein-bound Ca (mg/l) and P is total concentration of protein (g/l).

The Ca_b was evaluated from its balance in the system, given as

$$Ca_t = Ca_b + Ca_f \qquad (2)$$

where Ca_t was the total concentration of Ca (mg/l) and Ca_f the free calcium (mg/l).

Ca_t and P were determined analytically in the UF retentate and Ca_f was taken as being equal to calcium concentration on the UF permeate side (Ca_{perm})

$$Ca_f = Ca_{perm} \qquad (3)$$

The equations 1-3 are valid if no protein is transported through the UF membrane to the permeate and no calcium is absorbed by the membrane. In our case these conditions were satisfied by membrane saturation with Ca and by permeate check for the presense of protein during ultrafiltration.

Concentration of calcium in the solutions was calculated per 1 g of protein in order to be able to compare samples with slight differences in protein content. The Ca-protein binding extent was expressed in weight units rather than molar concentrations because of variable protein molecular association in the experimental pH range used (McKenzie, 1971). Individual binding experiments were duplicated. Data were analyzed using analysis of variance and Duncan's multiple range test (SAS, 1985).

Analytical procedures. Calcium was determined by atomic absorption (Brule et al., 1974) with the Perkin-Elmer 4000 atomic absorption spectrophotometer (Perkin-Elmer Corp., Norfolk, CT) using a hollow cathode lamp (type 3UNX/Ca) at 422.7 nm wavelength (air-acetylene flame). The standard solution of $LaCl_3$ was added to the samples (500 ppm La) to prevent phosphorus interference. The precision of the determination was 0.02 ppm Ca.

The protein content was determined by a Microkjeldahl method #47.021 (AOAC, 1975) using 6.38 protein conversion factor.

7.3. Results and Discussion

α-lactalbumin

The Ca binding patterns of α-la shown in Fig. 7-1 revealed strong pH dependence of the protein capability to bind Ca. It is immediately apparent, that the binding extent was enhanced by increasing pH. As Ca total concentration in the sample solutions increased, the linear portion of binding profile curves reached a final plateau. The levelling-off in the curves differed for individual pH and was considered to be the maximum binding potential. The calcium concentration at this point (mg Ca/g protein) was arbitrarily called saturation concentration (Ca_s). Further increase of Ca concentration in the solution had no effect on the amount of Ca uptake and the protein appeared to be saturated with calcium. The Ca_s increased with increasing pH and at pH 7.0 was approximately 15 mg Ca/g protein.

Dramatic increase of the amount of bound Ca observed at pH 4.0 compared to pH 3.5 (protein-bound Ca not detectable) confirmed the previously suggested reversible conformational transition of the protein caused by lowering of proton concentration and resulting in the increase of Ca binding

(Kronman et al., 1981; Permyakov et al., 1982). The additional increase in the Ca binding extent above the level established at pH 4.0 appeared to be governed by the increasing net protein charge as the pH was approaching more neutral values. The existence of two Ca binding sites on the α-la at neutral pH with different binding capability was suggested by Hiraoka et al. (1980) and confirmed recently by Bunzli et al. (1988).

β-lactoglobulin

Compared to α-lactalbumin the Ca binding profile of β-lactoglobulin was quite different (Fig. 7-2). There was no calcium binding to the protein at pH below 5.0. The calcium-β-lg binding became significant above the protein isoelectric pH. The observed level of bound Ca was the highest at pH 7, the upper limit of our experimental pH range.

Similarly to α-la the increase in Ca binding ability of β-lg appeared also to follow the increases in protein negative charge. The observed extent of bound Ca (3 mg Ca/g protein at pH 7) was much lower than the 6 mg Ca/g protein (pH 7.4) extrapolated from data of Carr (1953) and 5 mg Ca/g protein (pH 7) reported by Zittle et al. (1957). The lower Ca-β-lg binding appears to reflect the different ionic environment of our experimental system with the ionic strength of 0.08. The supressing effect of ionic strength on Ca binding potential of β-lg was reported (Baumy and Brule, 1988). To achieve β-lg binding maximum, a higher Ca saturation concentration was required compared to α-la. Furthermore, Ca_S increased steadily with increasing pH. As shown in Table 7-1 the change of Ca concentration from 10 mg Ca/g protein (Ca_s at pH 6) to 33 mg Ca/g protein yielded additional 25% increase in the amount of bound Ca at pH 7.0. The extent of Ca binding at such a high Ca concentration was not determined at pH 5.5 and 5.0 because the analytical errors in Ca determination exceeded the low level of the Ca uptake by the protein.

Effects of ionic strength

The Ca binding profile of both whey proteins, α-la and β-lg was further tested at four different ionic strengths, 0.04, 0.08, 0.12 and 0.16. In preliminary experiments it was found that doubling the respective protein concentrations did not affect Ca binding capability of both proteins. As shown in Figs. 7-3 and 7-4, the increasing ionic strength had an adverse effect on the amount of calcium bound to both α-la and β-lg. The observed binding maxima indicated the highest Ca binding at ionic strength of 0.04. At ionic strength of 0.16, the Ca uptake was the lowest and almost constant for the whole range of pH in the case of α-la.

Ca-protein binding in UF whey permeate

In order to confirm the observed Ca binding responses of both of the major whey proteins in an acid whey system, the extent of Ca binding was determined at various Ca levels in partially demineralized cottage cheese whey UF permeate and with separately prepared cottage cheese whey protein isolates. In parallel experiments using demineralized preparations from Sigma dissolved in the whey permeates no differences in calcium-binding response were evident and binding capacities of both types of protein preparations were essentially alike.

The Ca binding characteristics obtained at different Ca levels and evaluated in the pH range of 3.5 to 7.0 are shown in Fig. 7-5. The observed binding behaviour of both proteins resembled the binding exhibited in a simple buffer system. The differences in the binding potential at various Ca concentrations were insignificant. However, the distinction in Ca binding capacity was apparent between individual proteins. The α-la binding potential was almost twice as high as that of β-lg. When compared to buffer solutions, the recorded binding maximum of α-la was similar to that at ionic strength of 0.16 (Fig. 7-5); the pH increase did not have a substantial effect on the amount of α-la-bound Ca above the basic level established at pH 4.0. As in the buffer system, no interactions between β-lg and Ca were evident at or below pH 5.0.

7.4. Conclusions

This study confirmed that both α-la and β-lg have the ability to bind calcium. The binding potential was pH dependent and was further influenced by other factors such as ionic strength and calcium concentrations. Certain amount of calcium bound to α-la above pH 4.0 was independent of environmental factors of a given system, while the excess of α-la-bound calcium rose with increasing pH and was supressed at higher ionic strength. Below pH 4.0 Ca interactions with α-la were insignificant. The overall binding capability of α-la was about twice as high as that of β-lg.

No Ca binding to β-lg was evident below pH 5.0. Above the isoelectric point the extent of Ca-β-lg binding was highly dependent on physico-chemical characteristics of the system. However, the maximum binding was observed at pH 7.0, the limiting pH in our experimental range.

Although our model system using the cottage cheese whey UF permeate was not strictly equivalent to a complete whey system, the coincidental protein behaviour with respect to Ca binding in both the whey model and the simple buffer systems allows extrapolation to many whey processing situations. It appears that there are three major pH zones in processed cheese whey with apparently different magnitude of calcium-whey protein interactions: the pH region below 4.0 with no sign of such interactions; limited interactions between pH 4.0-5.5 due only to α-la; and substantial interactions above pH 5.5 due to both α-la and β-lg. At the pH approaching neutrality the total amount of Ca carried by β-lg is similar to that bound to α-la, because of the higher proportion of β-lg fraction in the total whey protein.

7.5. Tables and Figures

Table 7-1. Binding maxima of Ca to β-lg at various Ca concentrations[a].

Ca total (mg/g protein)	pH 5.0	5.5	6.0	6.5	7.0
	Ca bound (mg/g protein)[b]				
10	0.87 ± 0.08a[c]	1.09 ± 0.06b	1.63 ± 0.11c	2.32 ± 0.21d	2.39 ± 0.18d
20	nd[d]	1.09 ± 0.07b	1.85 ± 0.06e	2.54 ± 0.16f	2.92 ± 0.23g
33	nd	nd	1.87 ± 0.15e	2.51 ± 0.21f	3.04 ± 0.28g
44	nd	nd	nd	2.55 ± 0.12f	3.04 ± 0.20g

[a] ionic strength = 0.08

[b] means ± SD (n = 6)

[c] means within the same row and column sharing common letter are not significantly different at $P < 0.05$ level of significance

[d] not determined

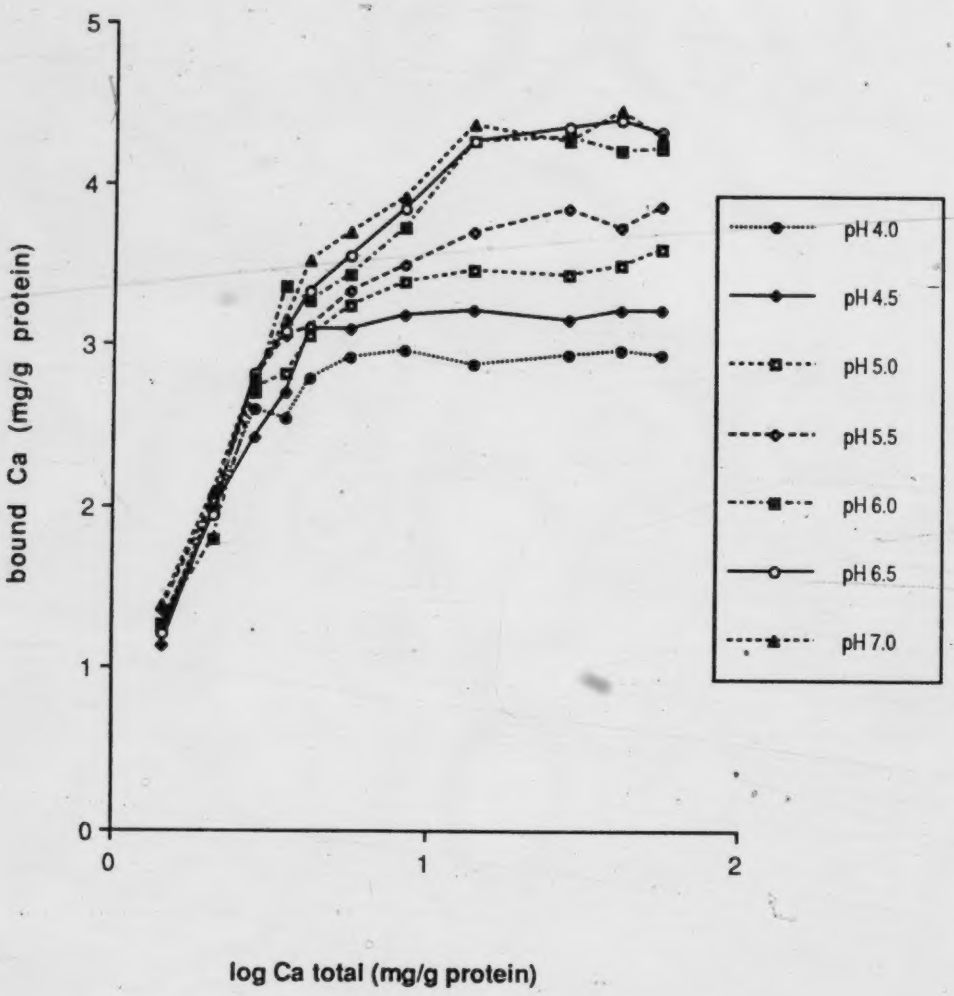

Figure 7-1. The Ca-α-la binding profile at various pH in buffer solution at ionic strength = 0.08. Absolute standard error of all data point means ± SD of three replicates from two parallel experiments was 4.5 ± 3.0%.

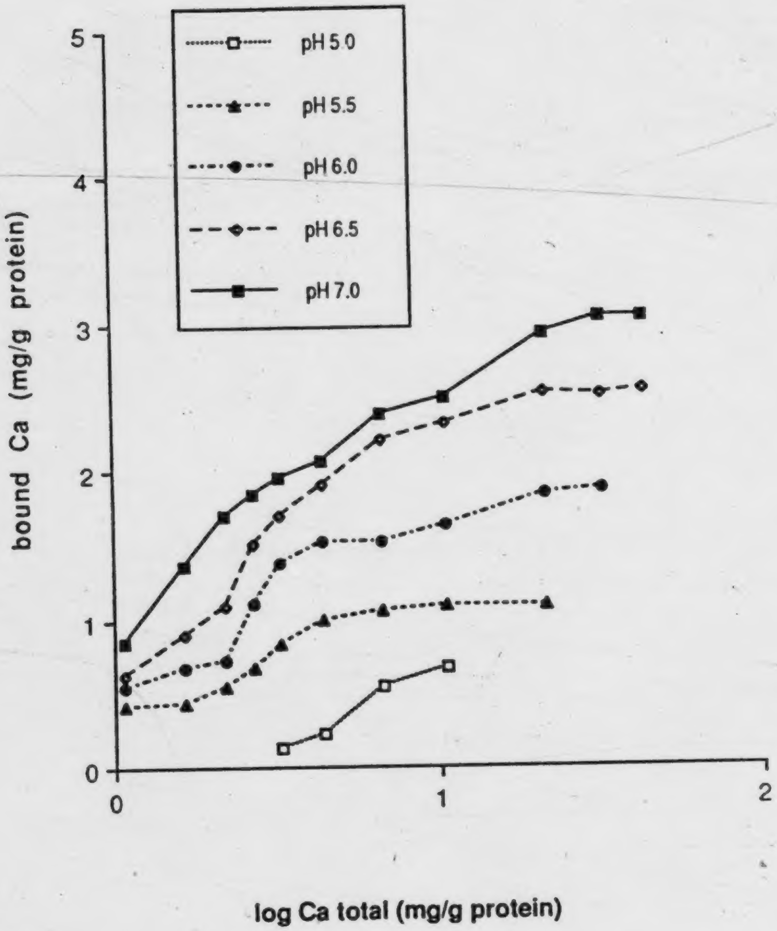

Figure 7-2. The Ca-β-lg binding profile at various pH in buffer solution at ionic strength = 0.08. Absolute standard error of all data point means ± SD of three replicates from two parallel experiments was 5.1±2.1%.

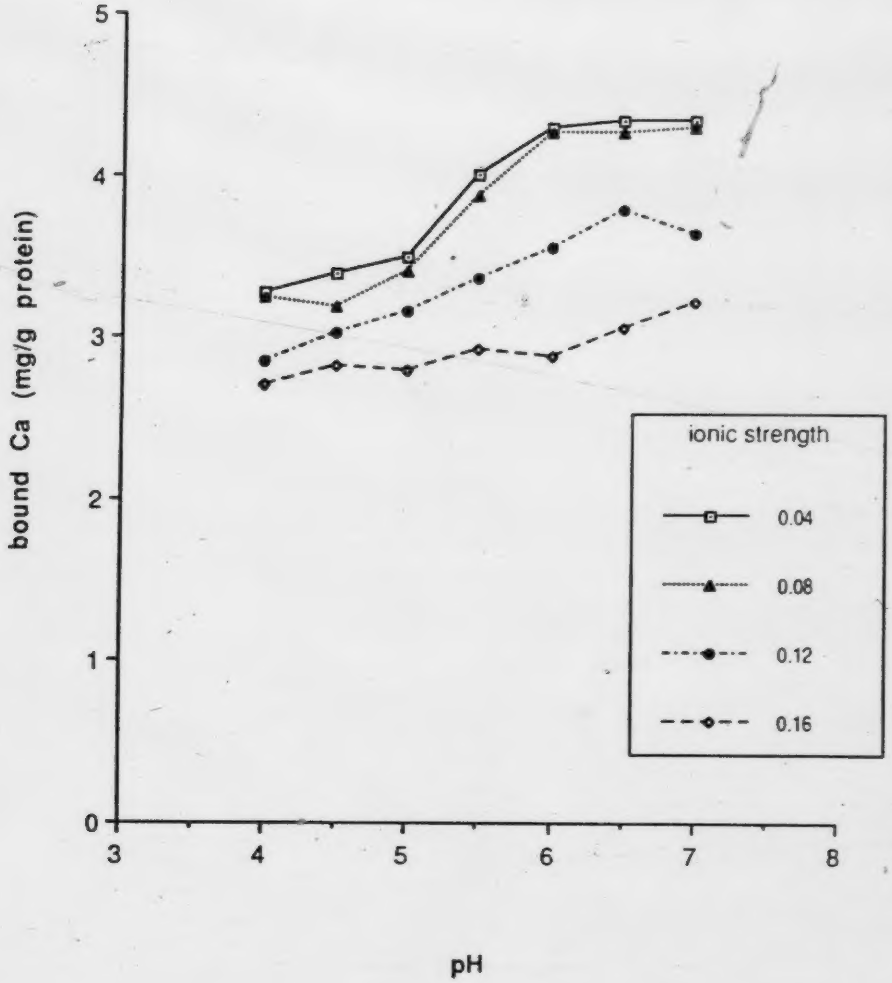

Figure 7-3. The effect of pH and various ionic strengths on binding of Ca to α-la. Absolute standard error of all data point means ± SD of three replicates from two parallel experiments was 4.7 ± 3.3%.

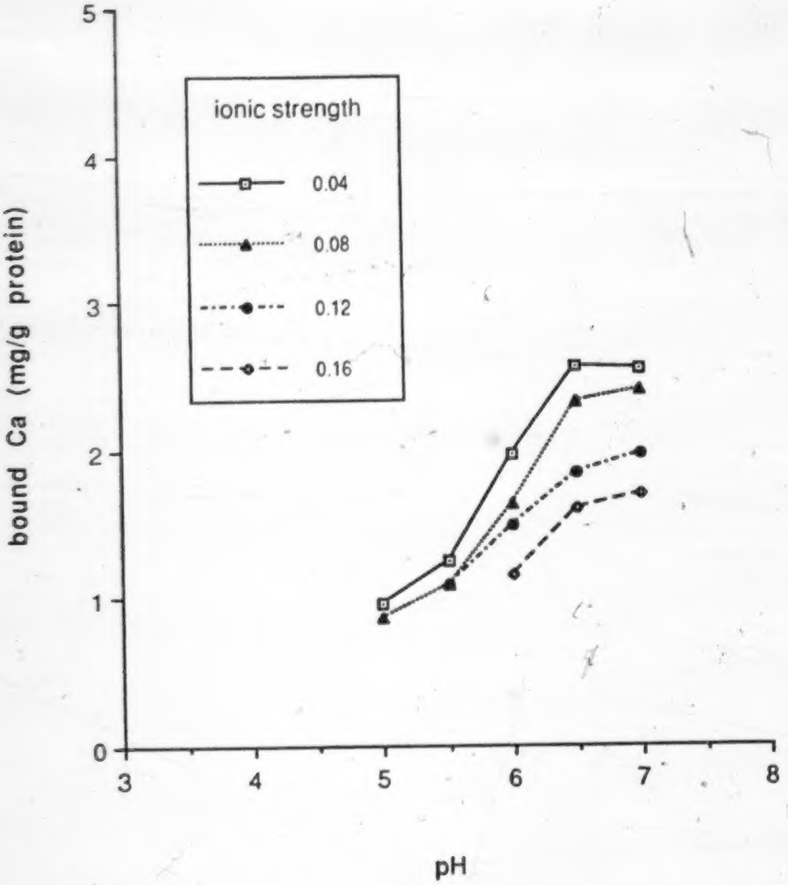

Figure 7-4. The effect of pH and various ionic strengths on binding of Ca to β-lg. Absolute standard error of all data point means ± SD of three replicates from two parallel experiments was 6.8 ± 2.7%.

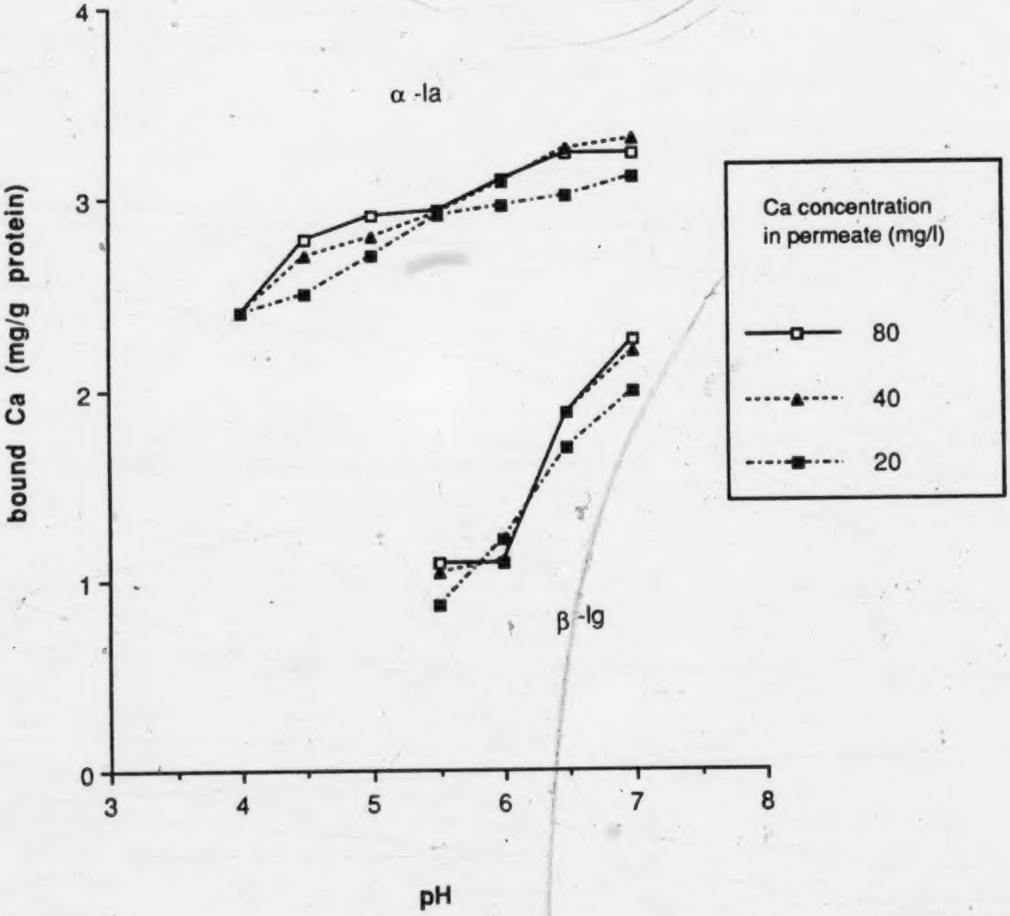

Figure 7-5. Binding of Ca to α-la (0.2%) and β-lg (0.4%) in UF permeate at various calcium concentrations. Absolute standard error of all data point means ± SD of three replicates from two parallel experiments was 5.8 ± 2.9%.

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8. HEAT INDUCED COAGULATION IN MILK WITH MODIFIED CASEIN : WHEY PROTEIN CONTENT[1]

8.1. Introduction

A milk clot formation in human stomach represents an important factor for ingestion of bovine milk. As revealed in a recent study (Buchheim et al., 1986), the gastric coagulation characteristics can be improved by partial replacement of casein with whey proteins in a ratio approaching that of human milk (Hambraeus, 1982). Incorporation of whey protein concentrates into fluid milk, suggested by McDonough et al. (1976), indicated nutritional benefits as well as good acceptability of such products.

Investigation of technological aspects of milks with modified casein : whey protein content and their application for manufacture of dairy products showed remarkable feasibility and attractiveness for dairy processors (Buchheim et al., 1986). However, Jelen et al. (1987) indicated that variations in thermal stability of these milks and derived products could be a limiting factor for some industrial applications. As heating is a major technological treatment in dairy processing, heat induced changes in whey constituents, especially proteins, may have a major influence on the quality of the products and the efficiency of the process.

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[1] An abbreviated version of this chapter has been published. P. Jelen and G. Patocka 1989. "Milk Proteins: Nutritional, Clinical, Functional and Technological Aspects", C.A. Barth and E. Schlimme (Eds.), Steinkopff Verlag, West Germany

The topic of thermal behaviour of whey proteins in terms of macroscopic physicochemical effects has been extensively reviewed by Hill (1986). However, only very limited information is available on thermal stability of fluid milk having unconventional ratio of casein : whey proteins produced by mixing regular milk with UF whey protein concentrates from different types of wheys. According to Kalab and Harwalkar (1973) thermal behaviour of such whey protein-containing systems is characterized by microstructural changes which might be indicated by the electron microscopy.

Therefore, the objectives of our investigation were: (1) to determine and compare the heat stability of ultrafiltration retentates (UFR) from acid and rennet type wheys and their subsequent blends with skimmilk; (2) to identify specific proteins in precipitates formed in those systems during heating up to 93°C; and (3) to illustrate observed macrostructural changes in heated modified milk with various casein : whey protein ratio by means of transmission electron microscopy (TEM).

8.1. Materials and Methods

The basic aspects of modified milk preparation were as described earlier (Jelen et al., 1987). Acid and rennet wheys were prepared in laboratory by cloting of regular pasteurized skimmilk (Palm Dairies, Edmonton) using lactic acid (85%) and single strength rennet (0.2 g/kg), respectively. Certain portions of wheys were decalcified by passing through an exchange column packed with BioRex 70 resin (BioRad Laboratories, Richmond, CA).

Ultracentrifuged milk serum was obtained according to Morr (1973) by centrifugation of skimmilk on a laboratory ultracentrifuge (L7-55, Beckman Instruments, Palo Alto, CA) at 140,000 x g for 2 hours. The wheys were clarified (Patocka and Jelen, 1989) on a Beckman J2-21 laboratory centrifuge and ultrafiltered on an Amicon UF cell (Amicon Corp., Danvers, MA) using 10,000 D PM-10 membrane to produce a 9.5% total solids content in the retentate, similar to skimmilk. The pH of acid and decalcified acid UFR was adjusted to 6.5 by 2N NaOH and 2N HCl, respectively. A portion of the rennet whey UFR was either regularly pasteurized (70°C/30 s) or thermized at 55°C for 30 min. The whey UF retentates were mixed with skimmilk to obtain milk blends with 0-100% UFR content. Similar experiments were carried out by mixing of UF permeates (UFP) and skimmilk to produce blends with 10-90% UFP content. Heating of 10 mL aliquots was in tubes submerged in a boiling water bath for 30 min. The come-up time for the samples to reach the maximum temperature of 93±1°C was about 2 min and was not included in the heating time. Heat stability was taken as a time needed for appearance of visually distinctive coagulation.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) before and after heating was carried out as in our previous work (Patocka et al., 1987). Before the application into the gel, precipitates isolated from heated milk were solubilized in the SDS reducing buffer (aqueous solution of 10% SDS, 0.05M Tris pH 6.8, 5% 2-mercaptoethanol, 5% glycerol).

Transmission electron microscopy. Heated milk samples cooled to room temperature were centrifuged at 95,000 x g on the Beckman L7-55 ultracentrifuge using 26.5 ml polycarbonate tubes. The pellets embedded in agar gel were cut into prisms of 2x2x10 mm, stored in 2% glutaraldehyde and subsequently subjected to the TEM. The electron microscopy work was carried out at the Electron Microscopy Centre, Agriculture Canada, Ottawa, by Dr. M. Kalab using methodology described earlier (Kalab et al., 1976).

Analyses. The protein (Nx6.38) and total solid contents were obtained by routine analytical methods (AOAC, 1975). The calcium was determined according to Brule et al. (1974) by atomic absorption with a Perkin-Elmer 4000 atomic absorption spectrophotometer (Perkin-Elmer Corp., Norfolk, CT) using acetylene-air flame at 422.7 nm wavelength.

8.2. Results and Discussion

Heat stability data for various skimmilk-cottage cheese whey protein retentate mixtures (Table 8-1) indicate that when the whey protein:casein ratio increased from 1:4, as in milk, to about 3:4 (i.e. mixtures of about 70% milk and 30% UFR), the heat stability was impaired. At these higher whey protein concentrations the heat induced β-lactoglobulin-κ-casein interaction did not have the usual stabilizing effect against the β-lactoglobulin precipitation. On the contrary, the higher calcium ion concentration in UFR seemed to induce casein co-precipitation and destabilization of the whole protein system, as demonstrated by the protein content (Table 8-1).

The SDS-PAGE showed differences between unheated and heated mixtures in a more striking way (Fig. 8-1). All protein patterns of the heated milk containing more than 50% cottage cheese whey UFR (slots 1b-5b) were characterized by the apparent decrease in intensity of all milk protein bands compared to protein profiles of the same samples before heating (slots 1a-5a; above this ratio, when heated milks became stable, the PAGE patterns of unheated and heated samples were identical (slots 6-8).

As evident from the electrophoretograms of solubilized thermal precipitates (Fig. 8-2) no casein was present in the precipitate isolated from heated cottage cheese whey UFR alone (slot 1). However, significant casein bands appeared in two following protein patterns (slots 2 and 3) of precipitates isolated from heated milks with the skimmilk/UFR ratio of 10:90 and 30:70 respectively.

The Ca content of the cottage cheese whey UFR decreased drastically after heating the pH 6.5 adjusted retentate and coagulum removal by centrifugation (Table 8-2). This may indicate a direct involment of calcium in whey protein thermal aggregation as suggested (Zittle et al., 1957; de Wit and Klarenbeek, 1984). In similar experiments with decalcified acid whey UFR, a partial precipitation was seen after 10 min of heating at 93^{o}C. Unheated or pasteurized rennet and UC sweet whey UFR appeared to be more heat resistant and no heat induced coagulation was observed in the conditions of the test. Furthermore, all combinations of milk and low Ca whey UFR were stable (Fig. 8-3), with the exception of mixtures with more than 50% unpasteurized rennet whey UFR. However, in this case the coagulation may have been due to the residual rennet activity in the UF concentrates. The heat stability of these milks was enhanced by either regular pasteurization (70°C/30 s) or thermization (55°C/30 min). This treatment, althought sufficient to innactivate rennet (Walstra and Jenness, 1984) does not induce substantial changes in stability of the milk protein system (Bernal and Jelen, 1985).

An alteration of total protein content within the same ionic environment of the modified milk by replacing the acid whey UF retentate with the acid whey UF permeate decreased the milk thermal instability. As indicated by heat stability data summarized in Table 8-3, all combinations of the cottage cheese whey UFP (pH adjusted to 6.5) with skimmilk (except 90:10 skimmilk/acid whey UFP blend) coagulated before reaching 93°C. When this whey UF permeate was decalcified and then incorporated into the skimmilk, no coagulation was observed in the whole range of experimental mixtures. Similarly, no sign of detrimental effect on heat stability was seen after incorporation of a rennet whey UF permeate. The reason for such differences in coagulation response to heat appears to be related to the calcium content, particularly regarding the availability of free calcium. Rennet whey UF permeate contained about 5 times less calcium than the UF permeate from acid whey (Table 8-3) with only a little ionic calcium (Demott, 1968; de Rham and Chanton, 1984). This could explain why the heat stability was unchanged after the addition of the rennet whey UF permeate to the skimmilk.

Microstructure of heated milk with variable casein : whey protein ratio prepared from different UF retentates

Without any milk addition, the acid whey UFR coagulated upon heating in the form of large submicroscopic aggregates of varying size (Fig. 8-4a). After casein incorporation these aggregates formed clusters typical for coagulated milk protein (Fig. 8-4b). With increased proportion of skimmilk in the blends (Fig. 8-4c), the globular and elongated aggregates produced upon heating were substantially finer. After decalcification, the microstructural pattern of acid whey UFR was characterized by the presence of very large globular particles (Fig. 8-5a). This is somewhat surprising in view of the recorded heat stability, since no coagulation was observed. After blending decalcified whey UFR with skimmilk the heat-induced protein aggregates appeared slightly smaller and more dense (Fig. 8-5b). With casein-whey protein ratio > 60:40 the observed microstructure (Fig. 8-5c) resembled regular heated milk with clearly distinguishable globular particles (Kalab, personal communication). However, the aggregates were much larger and looser when compared to the milk prepared from regular cottage cheese whey UFR (Fig. 8-4c). There were no obvious differences in microstructure between milk samples prepared from either pasteurized and unpasteurized sweet or UC whey UFR. When these retentates were heated alone, their visual appearance changed to resemble regular skimmilk. Buchheim and Jelen (1976) interpreted similar milkiness observed in heated demineralized whey as an indication of the presence of non-sedimenting microscopic whey protein aggregates. As illustrated in Fig. 8-6, heating of the UFR prepared from unpasteurized rennet whey produced large well defined protein aggregates with no signs of forming cluster chains (Fig. 8-6a). After incorporation of casein the aggregates became smaller and noticeably densely packed (Fig. 8-6b). With high casein content the aggregates of variable size appeared very loose and the observed microstructure resembled that of heated milk prepared with decalcified acid whey UFR but with a higher proportion of apparently smaller particles (Fig. 8-6c).

The remarkably large aggregate sizes observed in heated milks prepared from skimmilk and low Ca whey UFR appear to result from the changed ionic environment. Knoop and Peters (1976) observed that protein aggregates, after heating milk with reduced content of milk salts such as Ca citrate and phosphate, are larger due to lower electrostatic repulsion than heat aggregates formed in regular milk. However, when casein fraction becomes dominant in the total protein, whey proteins do not form aggregates alone but they are attached to casein micelles as appendages (Kalab et al., 1983).

8.4. Conclusions

The heat instability of milk with increased whey protein content appears to be due to calcium-modulated co-precipitation of both major whey proteins, β-lactoglobulin and α-lactalbumin, with casein resulting in destabilization of the whole casein-whey protein system. The skimmilk blends with UF retentates prepared from pasteurized rennet-type and UC wheys with genuinely low Ca-content, or from Ca-depleted acid-type whey were heat stable.

Comparison of the electron micrographs of heated milks with variable casein-whey protein content demonstrated major structural changes affected by the individual type of whey UFR used. Heating of skimmilk blends with decalcified acid whey UFR resulted in the formation of large but loosely structured protein aggregates. The size and shape of heat-induced aggregates in milks prepared from low calcium whey UFR were similar; however, resulting aggregates were more dense and compact. The large aggregates forming the cluster chains were characteristic for milk proteins precipitated during or after heating.

8.5. Tables and Figures

Table 8-1. Heat stability of mixtures of skimmilk and cottage cheese whey UF; heating time 30 min max at 93°C (come-up time excluded).

skimmilk/whey UF retentate (% v/v)	heating time to coagulation (min)	protein content* (%w/w)
0/100	0	0.85
10/90	0	0.81
20/80	2	0.91
40/60	4	0.92
50/50	8	1.02
70/30	>30	3.28
90/10	>30	3.20
100/0	>30	3.40

*centrifuged after heating ; average protein content in mixtures before heating was 3.23% (range 3.12 - 3.40%)

Table 8-2. Calcium levels and observed heat stability (time before coagulation at 93°C) of whey UFR in comparison to skimmilk.

	Calcium (% w/w)	Heat stability (min)
acid whey UFR (pH 4.5)	0.109	0
acid whey UFR (pH 6.5)	0.083	0
heated acid whey UFR[a] (pH 6.5)	0.028	n.a.
decalcified acid whey UFR (pH 6.5)	0.014	10
rennet whey UFR (pH 6.6)	0.037	> 30
ultracentrifuged whey UFR (pH 6.7)	0.055	> 30
skimmilk	0.114	> 30

[a] centrifuged supernatant

n.a. - not applicable

Table 8-3. Calcium content of whey UF permeates and thermal stability (time before coagulation at 93°C) of skimmilk/whey UFP blends.

	Calcium (%w/w)	thermal stability of milk blends (min)
cottage cheese whey UF permeate (pH 6.5)	0.088	coagulation in the whole range except 90:10 mix (>30)
cottage cheese whey UF permeate decalcified (pH 6.5)	0.004	all milk heat stable (>30)
rennet whey UF permeate (pH 6.5)	0.023	all milk heat stable (>30)
UC whey UF permeate (pH 6.5)	0.031	all milk heat stable (>30)

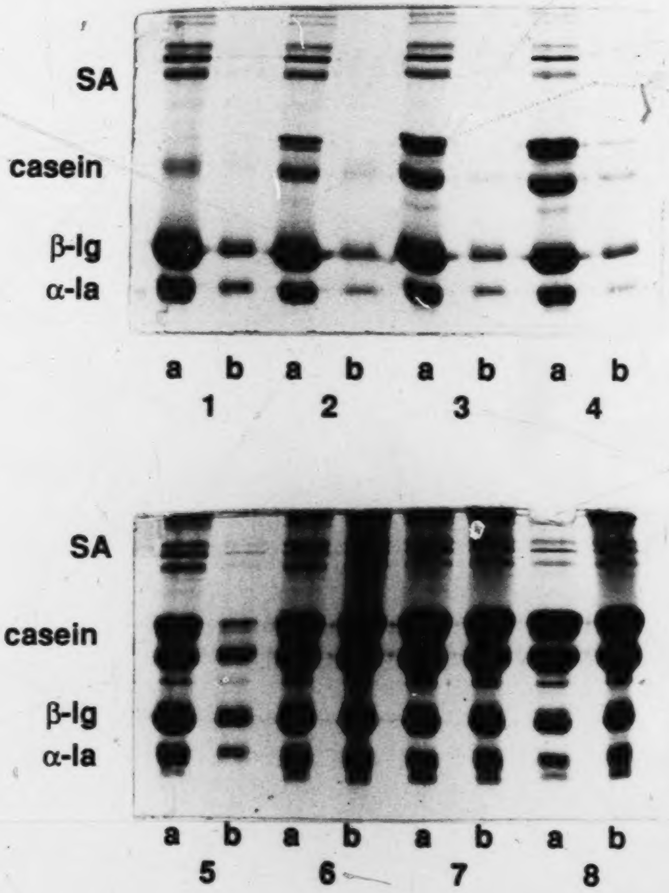
SA
casein
β-lg
α-la
a b a b a b a b
1 2 3 4
SA
casein
β-lg
α-la
a b a b a b a b
5 6 7 8

Figure 8-1. SDS-PAGE patterns of heat-unstable and heat-stable milk with varying whey protein content before (a) and after heating (b) at 93°C for 30 min. Skimmilk/cottage cheese whey UFR ratios: (1) - 0/100; (2) - 10/90; (3) - 20/80; (4) - 40/60; (5) - 50/50; (6) - 70/30; (4) - 90/10; (8) - 100/0.

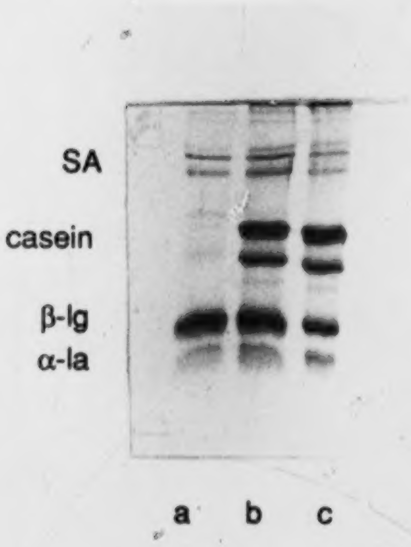

Figure 8-2. SDS-PAGE electrophoretograms of precipitates separated from milk with varying casein: whey protein content after heating at 93°C for 30 min. Skimmilk/cottage cheese whey UFR ratios: (1) - 0/100; (2) - 10/90; (3) - 50/50.

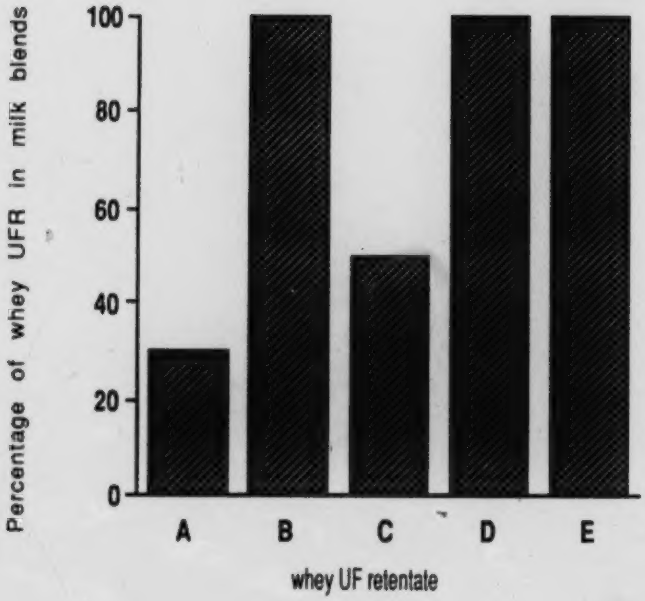

Figure 8-3. The maximum whey protein content of milk blends stable upon heating at 93°C for 30 min:

A - acid whey; B - decalcified acid whey; C - rennet whey;

D - pasteurized rennet whey; E - ultracentrifuged whey.

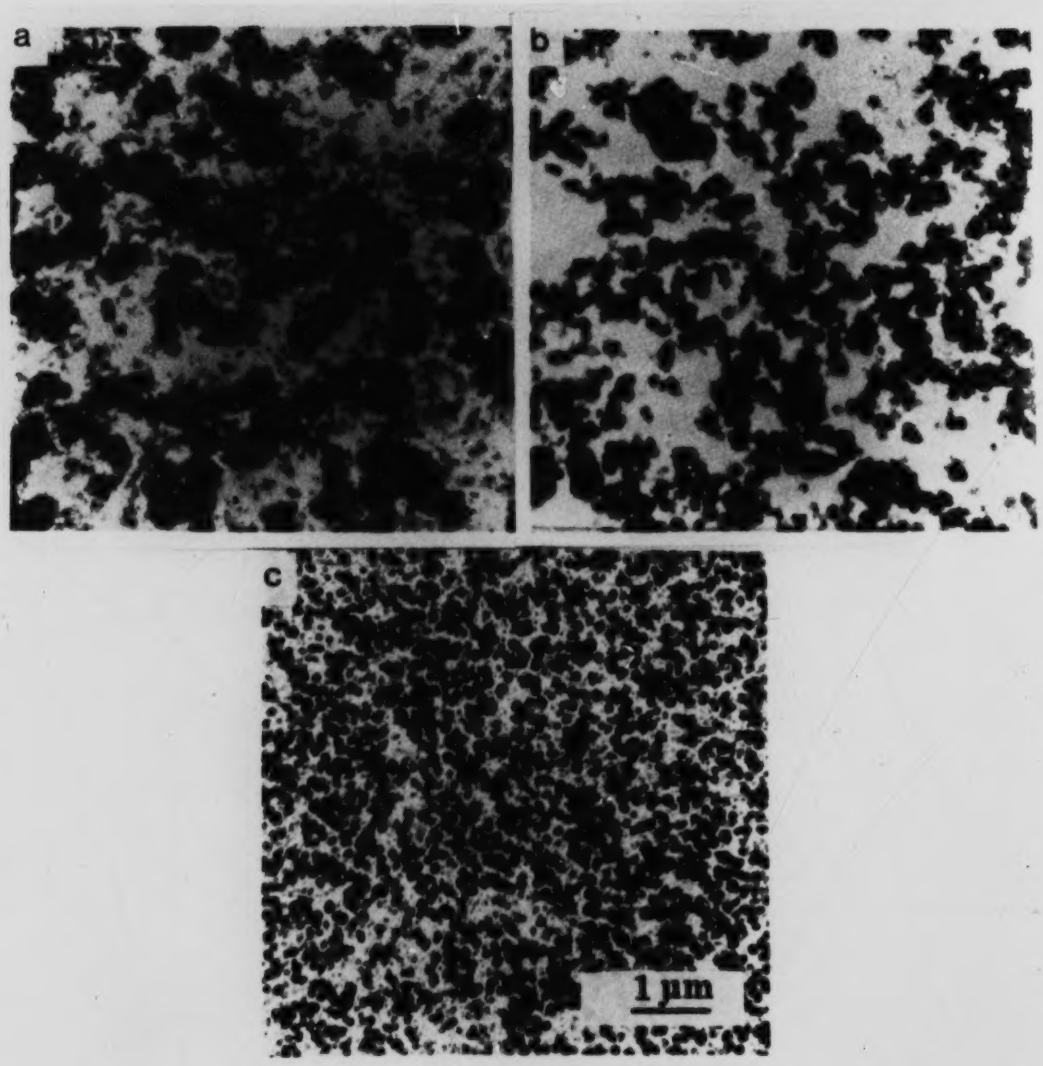

Figure 8-4. Transmission electron micrographs of cottage cheese whey UFR/skimmilk preparations after heating at 93^{O}C for 30 min: a) - whey UFR with no skimmilk; (b) - 10/90 skimmilk/UFR ; (c) - 70/30 skimmilk/UFR.

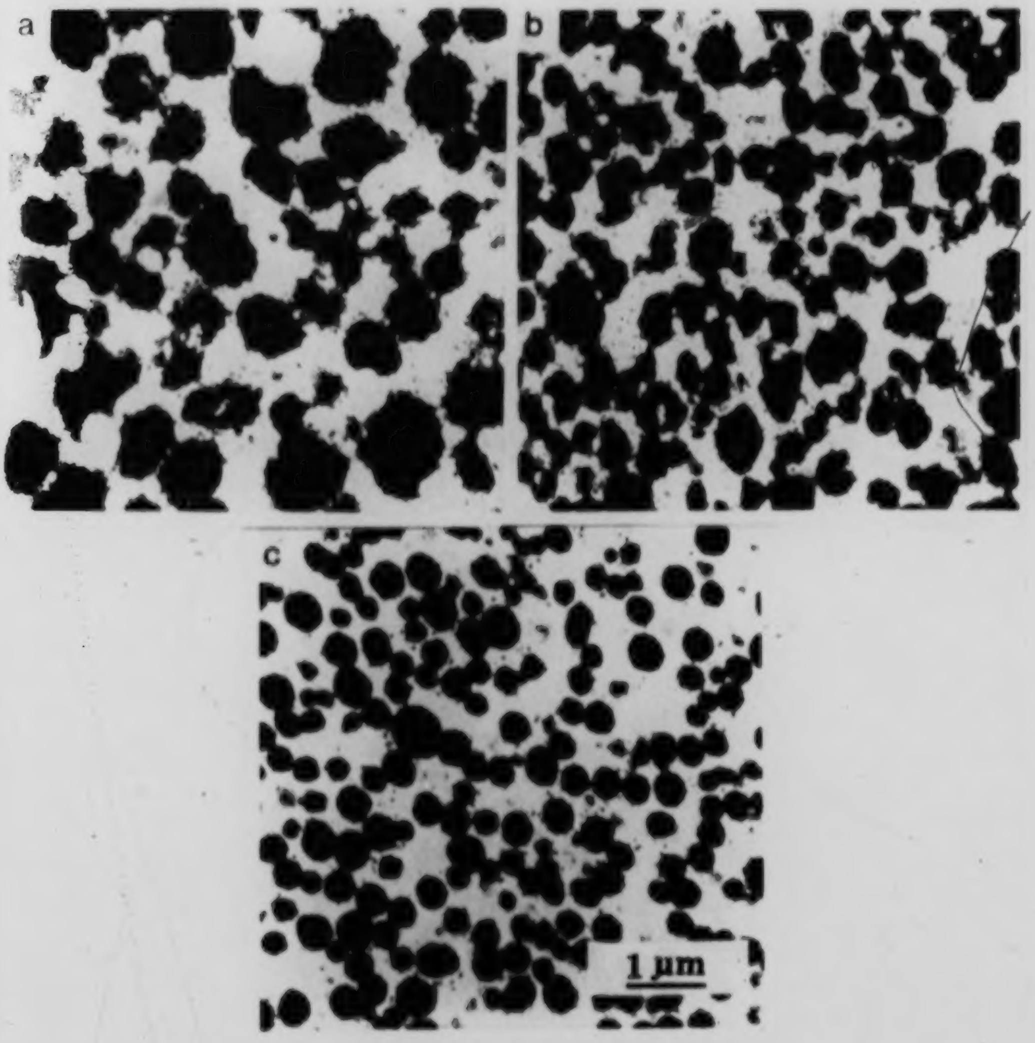

Figure 8-5. Transmission electron micrographs of decalcified cottage cheese whey UFR/skimmilk preparations after heating at 93^{o}C for 30 min: (a) - whey UFR with no skimmilk; (b) - 10/90 skimmilk/UFR ; (c) - 70/30 skimmilk/UFR.

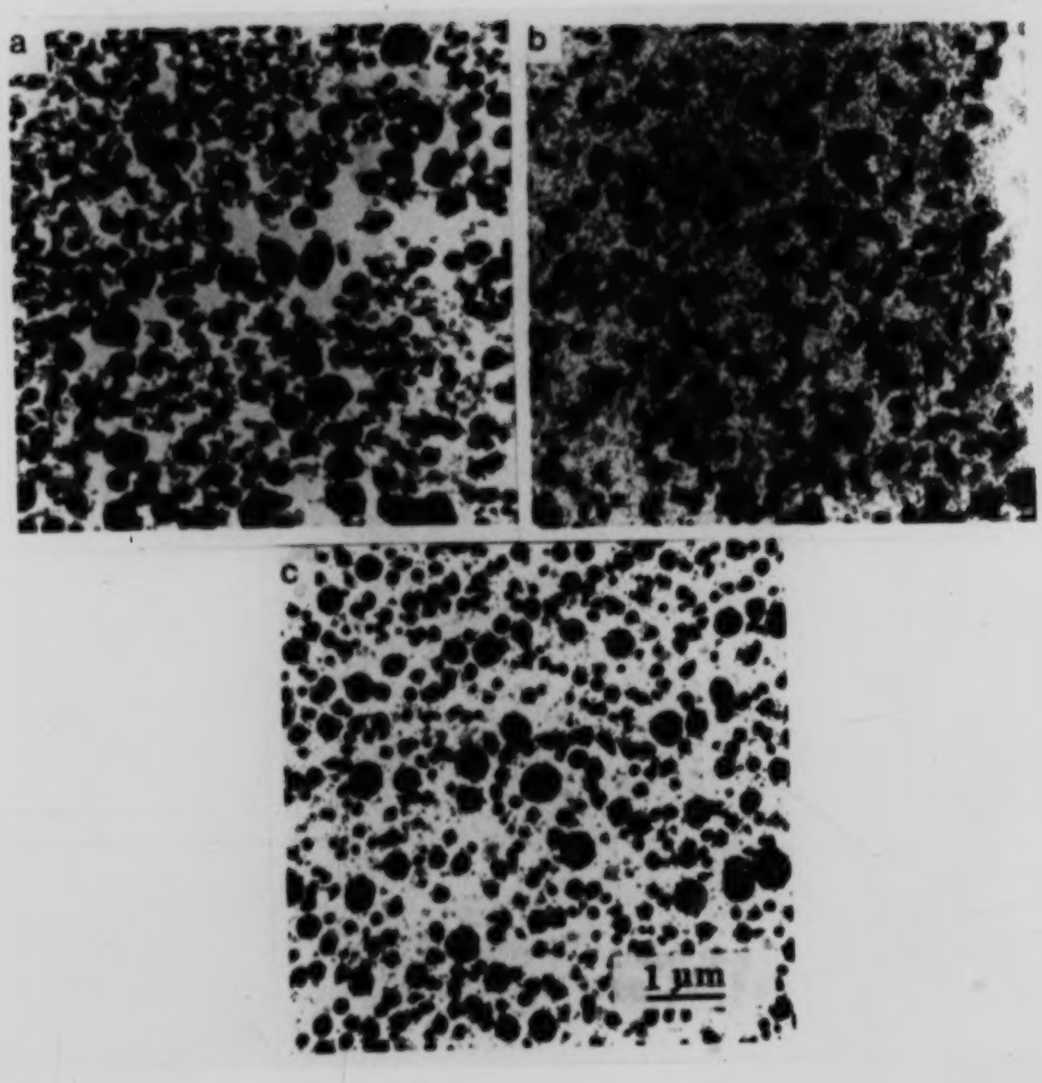

Figure 8-6. Transmission electron micrographs of unpasteurized rennet whey UFR/skimmilk preparations after heating at 93°C for 30 min: (a) - whey UFR with no skimmilk; (b) - 10/90 skimmilk/UFR ; (c) - 70/30 skimmilk/UFR.

8.6. References

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9. SUMMARY AND CONCLUSIONS

The results of these investigations provide new information leading to further understanding of the whey protein behaviour applicable to cheese whey processing. The major findings summarized in the following paragraphs indicate that the efficiency of the whey protein recovery processes and their applicability to various food systems may be improved markedly by physical and chemical pretreatment of whey, and that environmental conditions in various whey protein systems are the major determinants of whey protein behaviour upon heating.

9.1. Summary of research findings

The centrifugal clarification of cottage cheese whey appeared to be the simplest way to remove the residual casein, to maximize ultrafiltration rate of cottage cheese whey and to eliminate any possible interference of casein in further studies. The effectiveness of centrifugation for removal of residual casein fines was higher in comparison to whey clarification achieved by sedimentation. The turbidimetric measurements of absorbance correlated well with residual casein content and this proved to be a very simple, quick, and reliable method for the control of whey clarity. The ultrafiltration of centrifuged whey showed about 30% flux improvement compared to the whey ultrafiltered after 24h sedimentation.

The behaviour of proteins in cheese wheys acidified below pH 4 indicated a complete change of heat precipitation characteristics from labile to stable within a very narrow pH range. Electrostatic repulsion forces created at the low pH appeared to prevent the denatured protein from aggregating together and precipitating. There was a strong indication of the prominent role of calcium in the stabilization of whey protein upon heating. Heated sweet wheys showed a slightly lower resistance towards heat induced precipitation than acid wheys, apparently due to the lower total calcium content. After calcium removal by ion exchange, the transition pH of whey protein stability became identical for both sweet and acid wheys.

When investigated separately in the 3.5-7.0 pH range using regular or decalcified cottage cheese whey UF permeates, the individual major whey protein fractions (β-lg, α-la) showed significantly different responses to thermal treatment. The behaviour of β-lg generally followed the trends observed in whole wheys, with the protein being resistant to heat-induced coagulation at pH 3.7 in the regular permeate but precipitating at this pH in decalcified permeates. Beyond pH 3.8 heating 0.4% β-lg solutions resulted in rapid flocculation before reaching 93^{o}C up to the pH 6.5 regardless of the calcium content. In the pH range of 6.5-7.0 the reduction of calcium resulted in increased stability of the protein. The isolated α-la, in the absence of β-lg but with the Ca present, showed no sign of any precipitation up to the pH 5.0. Further pH increase resulted in protein precipitation from heated regular permeates. In decalcified permeates, this protein appeared completely resistant to heat coagulation in the whole pH range from 3.0 to 7.0. After slight increases in turbidity in α-la solutions observed on the onset of heating, no further changes followed.

The thermal behaviour of α-lactalbumin and β-lactoglobulin might be summarized as follows.

β-lactoglobulin

Below pH 3.5 the protein is apparently heat stable and its stability is independent of the calcium content. In the pH region of 3.6-3.9 characterized by a noticeable transition in β-lg heat precipitation behaviour, reduced calcium availability shifts the protein heat stability to the lower limit of this pH range. In the 4-6 pH zone β-lg precipitates upon heating to 92°C and this isoelectric precipitation is independent of calcium concentration. Close to neutrality, i.e at pH 6.5-7.0, the calcium appears to be again a leading factor governing the β-lg behaviour upon heating; in the absence of Ca, the protein thermal stability increases.

α-lactalbumin

This protein is heat stable in the pH range from 3.0 to 5.0. The stability is independent of calcium. Above pH 5.5 the thermal response of α-la becomes Ca modulated. The protein is heat stable in decalcified whey systems, but increased Ca availability results in the α-la insolubilization. In the presence of β-lg the heated α-la, regardless of pH, appears to follow precipitation characteristic of β-lg and may co-precipitate.

The thermocoagulation characteristics of isolated SA (investigated only in the acidic pH range of 3.5-4.5 using regular and decalcified cottage cheese whey UF permeate) showed unaltered protein stability up to the pH 4.2 in regular permeates and up to the pH 4.5 in decalcified permeates. However, in a model system containing a mixture of all three proteins simulating concentrations found in whey, some of the SA appeared to co-precipitate with the β-lg and α-la.

When the interactions of α-la and β-lg with calcium were studied in model solutions at pH between 3.5 - 7.0 and ionic strength ranging from 0.04 to 0.16, both proteins showed capability to bind calcium. However, the extent of the binding was markedly pH dependent. Below pH 4.0 interactions between calcium and α-la were insignificant. The amount of calcium bound to the protein increased at pH values approaching the neutral zone. The overall Ca binding capability of β-lg was about half of that of α-la. The first signs of a β-lg-Ca interaction were observed above pH 5.0. The increase of ionic strength had a negative effect on Ca-protein binding with both whey protein fractions. The analogy of calcium binding behaviour of α-la and β-lg in simple buffer solutions and model whey system allows to suggest the existence of three major pH areas with respect to whey protein-calcium interactions in processed cheese wheys: no such interactions below pH 4.0; limited interactions between pH 4.0-5.5 due only to α-la; and significant interactions above pH 5.5 due to both α-la and β-lg.

Investigations of heat stability of milk with increased whey protein content showed differences related to the use of ultrafiltration produced whey protein concentrates from cottage cheese or rennet types wheys. The heat instability appeared to be due to calcium-modulated co-precipitation of whey protein with casein. The precipitation patterns appeared to be that of a complete destabilization of the whole protein system observed in this modified milk upon heating. It appears that the high whey protein concentration eliminated the generally accepted stabilization effect of κ-cn-β-lg complexation. The mixtures of skimmilk and whey protein concentrates prepared from rennet-type wheys with genuinely low Ca-content and from a calcium-depleted acid whey were heat stable. As confirmed by transmission electron microscopy the change of ionic environment and reduction of calcium in particular resulted in the heat-induced formation of large, but loosely structured aggregates, nevertheless resistant to coagulation.

9.2. Significance for industrial processing

Whey clarification by centrifuging is industrially feasible because of its simplicity. The minimal processing time favors this technique against the commonly used sedimentation. The effectiveness of casein fines removal can be easily monitored by absorbance measurements. The method is suitable for routine control in industrial plants as indicated by its recent test application in a large dairy plant. The rate of cheese whey ultrafiltration can be significantly improved by complete clarification achievable only by centrifugation.

The measurements of turbidity can be also used as a rapid and reproducible method for evaluation of the extent of changes occuring in processed whey during heat treatment.

For certain food application, the susceptibility of whey proteins to heat induced precipitation is a defect limiting the whey protein use. In development of whey based beverages, the heat-coagulability of whey proteins is especially important as it may result in undesirable cloud and/or sediment formation. It can be recommended that industrial processes where whey protein coagulation upon pronounced heating is undesirable should be carried out at conditions not exceeding pH 3.5 whenever possible.

The role which calcium plays in various whey processing techniques has to be assesed with respect to a given technological specification. Calcium reduction or its complete removal appear to be highly beneficial for membrane separation. Nevertheless, in highly acidic conditions, insufficient calcium level in the whey may shift the pH required for transition from heat unstable to heat stable conditions to about 3.6 which may be too low for some consumer applications. The low free calcium content in wheys at pH values close to neutrality leaves whey proteins quite thermostable, which would be beneficial for manufacture of products requiring good solubility. However, this could be undesirable, if whey protein precipitation is required. In this case the pH has to be adjusted back to the lower pH range. It could be expected that whey proteins can serve as a carrier for small amounts of calcium incorporation into various food systems at neutral pH. However, no calcium would be associated with whey proteins below their isoelectric pH.

While earlier works recommended either sweet or acid whey as a suitable material for the manufacture of milk with increased whey protein content, our results indicate the limited usefulness of whey protein concentrates obtained from untreated acid type wheys. Incorporating such a material into the milk can be recommended only for dairy products where minimal heat treatment is required and heat instability may not be detrimental (pasteurized milk, cultured fluid milk products). Whenever severe heat treatment is a part of the processing technology such as manufacture of yogurt, ice cream, UHT milks, or even condensed and dried products, decalcification of acid whey is necessary to prevent protein insolubilization upon heating.

9.3. Recommendation for future studies

Future studies towards greater understanding of whey protein behaviour under various processing conditions are still necessary. In industrial cheese whey processing a great deal of information is essential for process optimization and attainment of desirable functional properties.

The effectiveness of the flux optimizing pretreatments has to be evaluated further for cheese whey ultrafiltration processes carried out on metallic membranes. The equipment containing this "third generation" membrane is now available. The data collected in this and future studies should be transferred into a general mathematical description of the process.

Preliminary experiments conducted outside the framework of this project showed that precipitation characteristics of whey proteins observed in whey or model systems seem to differ markedly in more complex food systems such as whey beverage containing fruit juice, where the additional heat-induced whey protein co-precipitation with certain fruit components (pectins, tannins) may occur. A whey based drink containing fruit juice was formulated for the final pH below the critical whey protein coagulation range. After UHT processing, immediately apparent strong cloudiness and heavy sedimentation developed during subsequent storage and this substantially reduced product acceptability. Because of the lack of available information, additional studies are required to identify this type of heat induced changes, to specify whether whey proteins, either as a group or as individual protein fractions, are involved together with fruit components and if so, to determine mechanisms of presumable interactions, particularly between whey proteins and pectin.

In spite of the information gained in this study, which indicates calcium as an important factor determining whey ultrafiltration rate as well as affecting thermal behaviour of whey proteins, understanding of the role of Ca in various whey systems is far from being complete. The results of this study confirmed the earlier observations that only certain fraction of total calcium, the free form, is responsible for any kind of activity in whey. Future investigations should be focused on precise determination of ionic calcium concentration and its role under conditions of various whey containing systems.

The whey demineralization using a selective ion-exchange technique was frequently employed in this study; its broader application in industrial whey processing could open new prospects for the development of technological processes for whey based products with modified mineral contents. Processes should be devoloped to create custom-tailored nutritional and dietary products demanded and favored by consumers, but not available on the market. However, additional research is needed to establish optimum levels of mineral content together with evaluation of functional properties of those products. The legal and nutritional aspects of such technologies and their specification for industrial process should be an inevitable part of further research.

The incorporation of whey UF concentrates into fluid milk proved to be (within certain limits) technologically feasible. Additional studies are recommended to specify the optimum milk-whey mixing ratio as well as to propose applicability of modified milk for manufacture of individual dairy products. In our recent experience, the milk with high whey protein content can be readily used for yogurt preparation. However, observed destabilization of the casein-whey protein complex upon heating in presence of excess whey proteins will require further proof. Attempts should be made to determine the mechanism of such changes.

Whey is now regarded as a valuable source of proteins and other nutrients. The availability of feasible whey processing technologies appears more important today when the shortage of milk could become very significant. There is still enormous scope of the problems preventing worldwide commercialization of whey processing. Nevertheless, any success in this research area could have a considerable impact in the modern dairy industry especially in view of the magnitude of current whey wastage.